D. discoideum as a model for host-pathogen interaction: Phagosomal proteome of *Legionella*-infected cells

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades einer

Doktorin der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von Olga Shevchuk
aus Krasnoyarsk, Russland

1. Referent: Professor Dr. Michael Steinert
2. Referent: apl. Professor Dr. Jürgen Bode
eingereicht am: 21.04.2008
mündliche Prüfung (Disputation) am: 10.06.2008
Druckjahr 2008

A man must have a certain amount of intelligent ignorance to get anywhere.

Charles Franklin Kettering

Acknowledgements

I would like to thank all of those who have contributed to this work. The research that has gone into this thesis has been thoroughly enjoyable. That enjoyment is largely a result of the interaction that I have had with my supervisor, colleagues and collaboration partners.

I would like to begin by thanking Professor Michael Steinert, my thesis advisor and mentor for the past five years. I thank him for all time and energy he has invested into my research, for emotional support during unsuccessful experiment time, for hours he has spent with me, discussing everything from research to career choices. My life has been enriched professionally, intellectually and personally by working with Michael.

My thank goes to all of the friendly Legionella team for their help and advice: Sebastian Jacoby, Frank Galka, Can Ünal, Eva Schunder, Christiane Albert-Weissenberger, Carina Wagner, Simone Werner, as well as people who worked with me in IMIB. Particularly, I would like to thank Christoph Batzilla, who supervised my initial research steps in the proteomic field and Sonja Hägele, who established the *Dictyostelium-Legionella* interaction model and provided a considerable background for this work. My apologies to the others who I have not mentioned by name, I am indebted to them for the many ways they helped me.

I would also like to thank our collaboration partners who have provided essential input in this project. I fell very privileged to have worked with Dr. Carmen Buchrieser and Dr. Susanne Engelmann. I thank them for showing me what it takes to be successful woman in science and for all their friendly advices during my PhD project time.

I am grateful to Prof. Dr. Michael Schleicher for valuable discussion of my scientific results and many interesting ideas.

And finally, this thesis was only possible because of the support of Prof. Dr. Jörg Hacker. I thank him for giving me the opportunity to work in his institute and for access to all his laboratories and facilities.

Table of Contents

1 Summary

Legionella pneumophila, the agent of Legionnaires' disease, replicates intracellularly within a specialized phagosome of human macrophages and protozoan host cells. The major mechanism of intracellular survival and replication is based on the ability of *Legionella* to reprogram the phagosome maturation.

The social soil amoeba *Dictyostelium discoideum* has been established as a host model for several human pathogens, including *Legionella pneumophila*. The complete genome sequence, the genetic tractability and the phagocytic characteristics of *Dictyostelium* all generate many opportunities for the study of host-pathogen interactions.

In this work we have established two *Dictyostelium* assays. First is a plaque assay, employed as a screening system for bacterial virulence. Plaque assay reveals weather or not the pathogen displays virulence either by evading amoeboid killing or actively killing *Dictyostelium*. Twelve different *Legionella* species, including sequenced strains, uncharacterized patient isolates and a *Legionella*-like amoeba pathogen were analysed for their virulence potential. Moreover, the presence of genetic determinants of *Legionella* virulence, such as flagellin, phospholipases and regulatory genes were tested by DNA-array experiments. We found that certain virulence determinants, including phospholipases and regulatory proteins are also present in non- *pneumophila Legionella* isolates.

The second screening assay was performed to isolate *Legionella* mutants, which are defective in the reprogramming of host phagolysosomal maturation. To accomplish this, *Dictyostelium* cells were first incubated with iron-dextran, which loades the lysosomes. The cells were subsequently infected with a *L. pneumophila* Corby transposon mutagenized library. After four rounds of enrichment it was possible to isolate the mutants which were unable to prevent host phagosomal acidification.

The host factors that regulate maturation of the phagosome are largely unknown. Therefore, a detailed characterization of the composition of the *Legionella*-containing phagosome (LCP) is important for a better understanding of molecular mechanisms taking place during infection. To investigate the properties of these organelles, we established a protocol for the isolation of *Dictyostelium* LCPs that are free of other intracellular organelles. This method consists of mechanical lysis of infected cells and production of a postnuclear supernatant. Elimination of lysosomal compartments, loaded by colloidal iron during infection, was carried out on MiniMACS separation columns. The mitochondrial contamination was removed by INT "heavy" labeling of these organelles, followed by fractionation in a discontinuous sucrose density gradient. Electron microscopy analysis of these phagosome preparations revealed very little endosomal, Golgi- or

plasma membrane, but did contain some mitochondrial contamination. The described method proved to be a valuable tool to characterize the vacuolar compartment occupied by *Legionella*, and has potential to be applied to other vacuole resident pathogens.

The isolated phagosomal proteins were analyzed by MALDI-MS. A total of 157 proteins were identified. Twenty-eight of the proteins have been implicated in cytoskeleton organization and signal transduction. Most of these polypeptides were also found in latex beads phagosomes, indicating that key proteins are conserved during phagocytosis. We have found several functional classes of proteins, which have not previously been associated with the *Legionella* phagosome. These include protein biosynthesis machinery, proteasomal proteins, proteins with oxidoreductase activity and other molecules of unknown function. Some of these proteins thought to be potential targets for *Legionella* secreted effectors. In addition, the detection of endoplasmatic reticulum (ER) resident proteins such as calnexin, calreticulin and protein disulfide isomerase confirmed that *Legionella* reside in an ER-derived compartment.

Comparative proteome analysis of phagosomes containing the pathogenic strain *L. pneumophila* Corby and the low-pathogenic strain *L. hackeliae* revealed specific differences. In particular, we were able to observe alterations in a set of proteins which manipulate host cell signalling pathways and cytoskeleton reorganisation. The most prominent candidates for further analysis of their role in *Legionella* virulence include Rho dissociation inhibitor, protein with proteinase C inhibitor activity, superoxide dismutase, cystein proteinase inhibitor and some of elongation factors. Rho dissociation inhibitor and protein with proteinase C inhibitor demonstrated a two-fold increase in phagosomes containing *L. hackeliae.* The superoxide dismutase, cystein proteinase inhibitor and certain elongation factors in opposite are present in greater amount in *L. pneumophila* Corby phagosomes. In the case of *L. pneumophila* Corby infection we have also observed degradation of phagosome-associated actin. This degradation could mediate the actin cytoskeleton reorganisation and prevent the fusion of pathogenic phagosomes with acidic organelles.

In summary, we present here, for the first time, isolation and detailed protein characterization of *Legionella*-containing phagosome. The suggested phagosomal model and analysis of phagosomal alteration provide a framework for studying *Legionella-Dicytiostelium* interactions. Moreover, certain prominent host factors were proposed for elucidation of their role in bacterial infection.

2 Zusammenfassung

Legionella pneumophila, der Erreger der Legionärskrankheit, repliziert intrazellulär innerhalb eines spezialisierten Phagosoms in humanen Makrophagen und in Protozoen. Die Bakterien können intrazellular überleben und sich vermehren, weil sie die Reifung des Phagosoms manipulieren.

Die soziale Amöbe *Dictyostelium discoideum* ist bereits als Modellorganismus für zahlreiche Humanpathogene, so auch *Legionella pneumophila*, etabliert. Da die komplette Genomsequenz und die phagozytischen Eigenschaften bekannt sind, eröffnen sich viele Möglichkeiten zur Durchführung von Wirt-Pathogen-Interaktionsstudien.

In dieser Arbeit haben wir zwei Dictyostelium-Assays etabliert. Ein Plaque-Assay ermöglicht ein Screening bezüglich bakterieller Virulenz. Dabei wird ein Pathogen als virulent eingestuft, wenn es nicht von Dictyostelium verdaut werden kann, oder wenn die Infektion sogar zum Tod der Amöbe führt. In diesem Plaque-Assay wurden 12 *Legionella* Stämme untersucht. Dies waren zum Teil bereits sequenzierte Stämme, aber auch uncharakterisierte Patientenisolate und ein *Legionella*-assoziierter Stamm. Darüber hinaus wurden Eigenschaften, die wichtig für die Virulenz von *Legionella* sind, in einem DNA-Array untersucht. Dabei handelt es sich um Gene wie das Flagellin, Phospholipasen und um Gene für regulatorische Proteine. Wir konnten zeigen, dass bestimmte Virulenzgene, wie zum Beispiel die Phospholipasen und regulatorische Proteine auch in nicht-pneumophila Isolaten vorkommen.

Der zweite Assay diente dazu *Legionella* Mutanten zu isolieren, die die Reifung des Phagolysosoms nicht unterbinden können. Dafür wurden *Dictyostelium* Zellen mit Eisen-Dextran inkubiert, was sich in den Lysosomen ansammelt. Dann wurden die Zellen mit Mutanten aus einer *Legionella pneumophila* Corby Transposonmutagenese-Bibliothek infiziert. Nach vier Zyklen der Anreicherung konnten Mutanten isoliert werden, die nicht mehr dazu in der Lage waren die Ansäuerung des Phagosoms zu verhindern. Die Identifikation und Charakterisierung dieser Mutanten ist ein zukünftiges Projekt.

Wenn *Legionella* in Wirtszellen eindringt, werden spezielle Phagosomen gebildet. Es ist nicht bekannt, welche Wirtsfaktoren zur Reifung des Phagosoms beitragen. Eine detaillierte Charakterisierung des *Legionella* beinhaltenden Phagosoms (LCP) ist wichtig, um den molekularen Ablauf der Infektion besser verstehen zu können. Um die Eigenschaften dieser Organellen zu untersuchen, wurde ein Protokoll zur Isolation von bakterienfreien *Dictyostelium* LCP etabliert. Diese Methode beinhaltet die mechanische Lyse von infizierten Zellen und die Produktion eines post-nuklearen Überstandes. Das Herausfiltern der während der Infektion mit Eisen beladenen lysosomalen Strukturen wurde mit Hilfe von MiniMACS Säulen durchgeführt. Mitochondriale Kontaminationen wurden durch INT „heavy“ Markierung und anschließende Fraktionierung in

einem diskontinuierlichem Sucrose-Dichtegradienten entfernt. In elektronenmikroskopischen Untersuchungen konnte bestätigt werden, dass die Phagosomenpräparationen nur sehr wenig endosomale, Golgi- und Plasmamembranen enthalten. Vereinzelte mitochondriale Kontaminationen waren dagegen sichtbar. Die beschriebene Methode ist dafür geeignet das *Legionella* spezifische Phagosom für eine weitere Charakterisierung zu isolieren. Weiterhin hat sie das Potential für andere Pathogene angewendet zu werden, die sich in Wirtsvakuolen aufhalten.

Die isolierten phagosomalen Proteine wurden mit Hilfe von MALDI-MS analysiert. Dabei wurden 157 Proteine identifiziert. 28 Proteine sind an der Zytoskelettorganisation und an Signaltransduktionswegen beteiligt. Die meisten dieser Proteine sind auch in Latex-beads haltigen Phagosomen zu finden.

Somit gibt es Schlüsselproteine, die während der Phagozytose konserviert sind. Dazu gehören Proteine der Biosynthesemaschinerie, proteasomale Proteine, Proteine mit Oxidoreduktaseaktivität und einige Proteine, deren Funktion nicht bekannt ist. Einige dieser Proteine könnten potentielle Zielproteine für durch *Legionella* sekretierte Effektoren sein. Zusätzlich konnte durch die Identifikation von Proteinen des endoplasmatsichen Retikulums (ER) gezeigt werden, dass sich *Legionella* in einem Kompartiment aufhält, welches vom ER abstammt. Zu diesen Proteinen gehören unter anderem Calnexin, Calretikulin und Proteindisulfidisomerasen.

Vergleichende Proteomanalysen von Phagosomen mit pathogenen und schwach-pathogenen *Legionella* Stämmen ließen spezielle Unterschiede erkennen. So konnten wir Unterschiede bei einer Reihe von Proteinen feststellen, die die Wirtszellsignalwege manipulieren können und in den Zytoskelettumbau eingreifen. Die vielversprechendsten Kandidaten für eine zukünftige Analyse sind ein Rho- Dissoziationsinhibitor und ein Protein mit Proteinase C Inhibitor Aktivität. Diese Proteine kamen in großer Menge in Phagosomen mit schwach-pathogenen *Legienellen* vor. Die Superoxiddismutase, ein Cysteinprotease-Inhibitor und einige Elongationsfaktoren sind dagegen nach der Infektion mit Pathogenen in größeren Mengen zu finden.

Wir konnten im Falle einer Infektion mit *Legionella pneumophila* die Degradation von Phagosom-assoziiertem Aktin feststellen. Diese Degradation könnte zur Reorganisation des Aktin-Zytoskeletts führen und so die Fusion der pathogenhaltigen Phagosomen mit den ansäuernden Organellen verhindern.

Wir haben im Rahmen dieser Arbeit eine neue Methode etabliert, welche die Isolierung und anschließende detailierte Charakterisierung der Proteine im *Legionella*-haltigen Phagosomen ermöglicht. Das erarbeitete phagosomale Modell und die Analyse der Proteine im *Legionella*-haltigem Phagosom sind eine wichtige Basis für *Legionella-Dictyostelium* Interaktionsstudien. Weiterhin ist es jetzt auch möglich, die Rolle von Wirtsfaktoren während der bakteriellen Infektion zu bestimmen.

3 Introduction

3.1 *Legionella*

3.1.1 Legionellosis

Legionellosis is an infection caused by the bacterium *Legionella pneumophila*. The disease has two distinct forms: Legionnaires' disease, the more severe form involving a potentially fatal atypical pneumonia, and Pontiac fever, a milder form limited to flu-like symptoms. Legionnaires' disease acquired its name in 1976 when an outbreak of pneumonia occurred among participants in a convention of the American Legion in Philadelphia (Fraser et al. 1977). Although the exact source of this outbreak was not completely elucidated, the etiological agent was determined to be a previously unrecognised pathogen, named *L. pneumophila* (Brenner et al. 1979).

Humans become infected with *Legionella* after inhalation of contaminated aerosols from equipment that disperses water, such as showers, whirlpools or air condition systems. Legionnaire's disease most often affects middle-aged and older persons, particularly those who smoke, have chronic lung disease or those undergoing treatment involving cytotoxic chemotherapy. Pontiac fever, on the other hand, most commonly occurs in humans who are otherwise healthy. However, as person-to-person transmission of *Legionella* has never been demonstrated, humans have been inconsecutive for evolution of *Legionella* virulence (Molofsky and Swanson 2004). Once inside a human host, incubation may take up to two weeks. Initial symptoms are flu-like, including fever, chills, muscles aches and dry cough. Advanced stages of the disease affect the gastrointestinal tract and the nervous system. X-ray radiography usually reveals considerable lung damage with patchy infiltrated regions. Other advanced symptoms of pneumonia may also be present.

Since the clinical presentation is not specific for *Legionella* infections, microbiological and molecular biology diagnostic methods, such as urinary antigen detection and real time PCR of serum samples are currently used to detect Legionnaires' disease (Diederen et al. 2006; Diederen et al. 2007; Garcia-Vidal and Carratala 2006). Patients with Legionnaires' disease always require antibiotic treatment, following laboratory confirmation of the diagnosis. *L. pneumophila* is susceptible to many antibiotics, such as aminoglycosides, ß-lactam antibiotics and their derivatives *in vitro*. However, these drugs are often ineffective in treating Legionnaires' disease, due to the intracellular nature of this pathogen. The primary goal in the antimicrobial therapy is to prevent detrimental intracellular growth of *Legionellae* in alveolar macrophages (Jonas et al. 2000). Therefore, the drugs of choice include macrolides (erythromycin, clarithromycin, or azithromycin) and fluoroquinolones (ciprofloxacin, levofloxacin, or moxifloxacin) which possess the ability to

achieve intracellular levels and demonstrate high *in vitro* activity against *Legionella* (Mandell et al. 2003; Yu et al. 2004). The recommended treatment duration with these drugs is about three weeks.

In the United States of America *Legionella* affect 8 000 to 18 000 individuals per a year. In Germany 475 (554) cases of Legionellosis were reported in 2004 (2005). However, the total number of actual cases is presumed to be much higher. The mortality rate of this disease is up to 20 % (Stout and Yu 1997).

3.1.2 General characteristics of *Legionellae*

Legionella is an aerobic rod-shaped, Gram-negative bacterium, which is 0.5 µm in diameter and 1 to 2 µm in length. Most species are motile by means of one or more polar flagella. The pathogen utilizes amino acids as main carbon and energy sources (Tesh et al. 1983) and stores intracellular energy as poly-3-hydroxybutyrate (PHB). PHB is a homopolymer of 3-hydroxybutyric acid, which the bacteria accumulate during unbalanced growth to promote long-term survival under conditions of starvation (James et al. 1999). *Legionella* is able to survive under conditions of chlorination, UV radiation and low pH (King et al. 1988). *In vitro*, the growth of the organism depends on the presence of L-cysteine and iron in special media. The optimal temperature for bacterial growth under laboratory conditions is about 35 ºC and the optimal pH is 6.9±0.4 with generation times of 4-6 hours (Edelstein 1981; Feeley et al. 1979). However, in biofilms, the bacteria are able to survive temperatures above 60 ºC and pH up to 8.0.

The side-chain of the cell wall carries the bases responsible for the somatic antigen specificity of these organisms. The chemical composition of these side-chains both with respect to components as well as arrangement of the different sugars determines the nature of the somatic or O antigen determinants, which are essential for serological classification of *Legionellae*. Although 70 *Legionella* serogroups have been identified among 48 species at the moment, *L. pneumophila* which consist of 16 serogroups is responsible for most cases of Legionellosis. *L. pneumophila* serogroup 1 alone is responsible for 70-90 % of cases (Swanson and Hammer 2000). *Legionella micdadei* is the second most common species causing respiratory disease. In addition, a large number of *Legionella*-like amoebal pathogens have been described (Adeleke et al. 1996).

3.1.3 Ecology and host range of *L. pneumophila*

L. pneumophila is found ubiquitously in fresh water environments, where it exists planktonically, as a part of biofilm formation or within protozoan hosts (Harb et al. 2000). This bacterium is able to replicate within a number of amoebae, as well as within mammalian cells. It is therefore classified as facultative pathogen. Epidemiological, cell biological and genetic studies all show that amoebae have played the role of an evolutionary incubator for the emergence of *L. pneumophila* as an opportunistic pathogen of alveolar macrophages (Swanson and Hammer 2000). First, and

especially after encystation, amoebae provide a protection for *Legionellae* against adverse extracellular conditions. Secondly, the fact that *Legionellae* are able to replicate within amoebae indicates the presence of adequate nutrition for this bacterium within amoebae. Thirdly, adaptation of *Legionellae* to survive within amoebae may have primed this pathogen to infect human cells.

Legionellae are capable of infecting and multiplying in a wide range of hosts: 13 species of amoeba, two species of ciliated protozoa, mammalian macrophages and epithelial cell lines. It appears that *L. pneumophila* has the most extensive host range among *Legionellae*. Other species of *Legionellae* are limited in the types of host cells they can infect (Fields 1996). *Legionella* virulence has been extensively studied on protozoan host-models as well as on mammalian cell lines and different animal models. The studies provided the experimental basis for investigating the role of host immune responses in the pathogenesis and for drug therapies, suggested for Legionnaires' disease treatment. The susceptibility of five laboratory animal species (mice, guinea pigs, rats as well as syrian and chinese hamsters) to infection with different *Legionella pneumophila* species has previously been tested. Based on clinical manifestations, pathological macroscopic changes and detection of *Legionellae* numbers in animal organs, it was assumed that guinea pigs are the most suitable experimental animals for investigations and the diagnosis of Legionellosis (Spalekova and Danihel 1994). Coming back to the role of amoebae in *Legionella* virulence, it is interesting to notice that mice inoculated with a mixture of bacteria and amoebae develop a more severe disease than those infected with either *L. pneumophila* or *Hartmannella vermiformis* alone (Brieland et al. 1996; Cirillo et al. 1999). It was suggested that protozoa worsen the lung damage caused by *L. pneumophila* infection by amplifying the number of *Legionella* thereby increasing the dose of bacterial cytotoxin(s) produced in the lung. Alternatively, amoeba might contribute to pathogenesis indirectly, by triggering a hyperactive, ineffective immunoresponse (Swanson and Hammer 2000). Histological studies of lung material obtained from infected guinea pigs revealed that the fate of intracellular *L. pneumophila* depends on their phagocytic hosts. More than 95 % of the bacteria were intact within macrophages, whereas most *Legionella* within neutrophils were degraded (Katz and Hashemi 1982). A number of further studies have demonstrated the ability of *Legionella* to replicate within primary macrophages and a wide variety of cell lines, including some derived from monocytes, fibroblasts and epithelial cells (Fields 1996).

3.1.4 Intracellular life cycle of *L. pneumophila*

The life cycle of *L. pneumophila* in amoeba strongly resembles that observed in macrophages (Fig.3.1). The initial interaction between an intracellular pathogen and the host cell is mediated by the attachment of bacterial adhesins to a surface receptor on the host cell. This ligand-receptor binding precipitates signal transduction processes which in turn initiate polymerisation of actin, leading to phagocytic uptake.

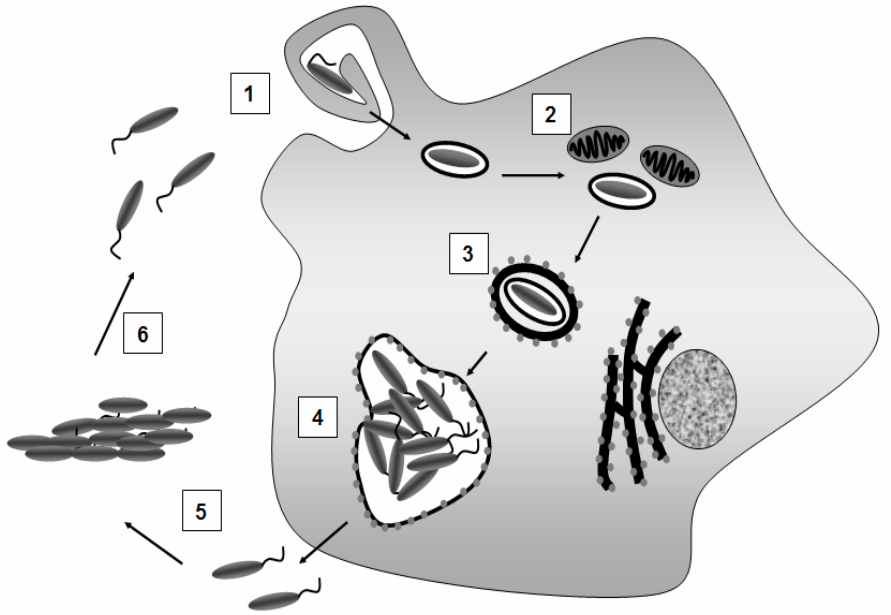


Fig. 3-1 Intracellular life cycle of *L. pneumophila*
1. Invasion. Free-swimming transmissive *L. pneumophila* are engulfed by phagocytic cells (amoebae or alveolar macrophages).
2. *L. pneumophila* establish vacuoles that provide protection from lysosomal digestion.
3. *Legionella*-specific vacuole recruits ER and ribosomes.
4. When nutrients are favourable, intracellular bacteria repress transmission traits and activate pathways that promote replication.
5. The phagocyte host is lysed, and the microbes are released into the environment.
6. *L. pneumophila* that do not encounter a new phagocyte establish biofilms in both water systems and ponds, where they are resistant to biocidal agents. When planktonic microbes encounter a new phagocyte, the cycle begins anew.

Two attachment mechanisms have been described for *Legionella*. The first is opsonic, used by human-derived phagocytic cells, and the second is non-opsonic, used by protozoan hosts. For example attachment to *H. vermiformis* is mediated by adherence to a protozoan receptor galactose/N-acetylgalactosamine (Gal/GalNAc) lectin. This lectin was described to have similarity to the β2 integrin-like Gal/GalNAc lectin of *Entamoeba histolytica* (Harb et al. 1998), which undergoes tyrosine phosphorylation upon ligand binding and subsequently results in recruitment and rearrangement of the cytoskeleton.

Following attachment bacteria are taken up by host cells via conventional or coiling phagocytosis, in which a bacterium is surrounded by a multilayer coil-like membrane structure. After phagocytosis, *L. pneumophila* can be found in a membrane-bound phagosome. During the first hours following infection these organelles are surrounded by smooth cytoplasmic vesicles and mitochondria. As the *L. pneumophila*-containing compartment matures, ribosomes begin to associate with the vacuole and the smooth cytoplasmic vesicles become less abundant (Abu Kwaik 1996; Derre and Isberg 2004; Horwitz 1983; Kagan and Roy 2002; Robinson and Roy 2006). After eight hours, the bacteria-containing compartment is completely surrounded by ribosomes, and *Legionella* begin to replicate. Several electron micrograph observations have shown that these

smooth cytoplasmic vesicles are actually vesicles derived from the host endoplasmatic reticulum (ER). Using ER markers, such as glucose-6-phosphotase, protein disulphide isomerase (PDI) and proteins having the ER-retention signal lysine-aspartatic acid-glutamic acid-leucine (KDEL), it was shown that ER fuses with *Legionella*-containing vacuoles as early as 5 min after uptake (Robinson and Roy 2006). The authors propose that fusion with ER may be important for delivery of peptides and other nutrients into the lumen of *Legionella* phagosome that are used for bacterial multiplication. Establishing a replicative phagosome by avoiding the host endocytic pathway is a characteristic feature of *L. pneumophila.* This *Legionella*-containing phagosome (LCP) excludes endocytic markers, like lysosomal associated membrane glycoproteins, LAMP-1 and LAMP-2, as well as the lysosomal acid protease Cathepsin D (Clemens and Horwitz 1995). Nevertheless, at late times of infection replicative *L. pneumophila* can reside in an acidic environment that possesses cathepsin D, indicating a fusion of the replicative vacuole with lysosome and acidification of the phagolysosome (Sturgill-Koszycki and Swanson 2000). At 24 hours post infection, the nutrients within the phagosome are depleted and bacteria switch to a highly resilient and infectious phenotype, characterized by sodium sensitivity, osmotic resistance, motility and cytotoxicity (Molofsky and Swanson 2004). The killing and lysis of macrophages, monocytes and epithelial cells occur in two phases. During early stages of infection *Legionella* induces apoptosis, a strictly regulated suicide program of the cell, which involves the activation of a family of cysteine proteases (caspases) that subsequently lead to DNA fragmentation of the host cell (Abu Kwaik et al. 1998). During late stages of intracellular replication, *Legionella* causes necrosis of its host cell, apparently by inducing pore formation. Based on cytotoxicity assays, confocal laser scanning microscopy and electron microscopy of mutants defective in pore-forming activity, it was concluded that this activity of *L. pneumophila* is not required for phagosomal trafficking or for intracellular replication. However, it is expressed upon termination of bacterial replication and essential to induce cytolysis of infected macrophages to allow egress of intracellular bacteria. In addition, it plays a major role in pulmonary immunopathology *in vivo* (Alli et al. 2000; Gao and Kwaik 2000).

Swanson and Molofsky postulated that biogenesis of a *Legionella* replicative vacuole is determined by the developmental state of the bacterium. When nutritions are exhausted, uncharged tRNAs activate the RelA, a guanosine 3', 5'-bypyrophosphatesynthetase, which subsequently causes an increase of ppGpp. This accumulation of ppGpp coordinates entry to the transmissive phase by repression of multiplication, but enchansing trait promoting transmission to a new host (Molofsky and Swanson 2004). The same research group assumed that biogenesis of intracellular *Legionella* strongly correlates with growth of the bacterium in broth culture. In particular, when broth culture enters the stationary phase, the bacteria behave like in macrophages before release and coordinately express the factors for induction of phagocyte necrosis, motility, resistance to the stress of UV light, heat, osmotic stress and nutrient limitation factors. Despite the indisputable advantages of

Legionella broth, the bacterial progeny that emerge from amoebae are more infectious and seem to be more suitable for study of *Legionella* pathogenesis (Cirillo et al. 1999).

3.1.5 Virulence determinants of *L. pneumophila*

A multitude of *L. pneumophila* virulence factors have been described in recent years. Most of them affect the ability of the bacterium to grow and survive within blood monocytes, alveolar macrophages, or free-living protozoa. Secretion systems play a pivotal role in virulence of *Legionella*. They deliver bacterial effector molecules to their site of action. To date, *L. pneumophila* has two major secretion systems known to be involved in pathogenesis. These are the type II (Lsp) and type IVB (dot/icm) secretion systems. The elucidation of the role of additional secretion systems is in progress.

3.1.5.1 Type I and type II secretion systems

A putative type I secretion system, which shows significant similarity to the respective systems of *V. cholerae* and *E. coli* was recently described. It is present in multiple *L. pneumophila* strains (Jacobi and Heuner 2003). This system is composed of six genes (*lss X, Y, Z, A, B,* and *D*) and was designated as Lss (*Legionella* secretion system). The function of the system has not been analysed so far, but it seems to be specific to *L. pneumophila* strains.

The type II secretion system (T2SS) of Gram-negative bacteria transports proteins across the outer membrane. The prerequisite transport across the inner bacterial membrane occurs either via the secretory (Sec) or via the twin-arginine translocation (Tat) pathway. While the Sec pathway transports unfolded proteins, the Tat pathway transportes folded proteins across the cytoplasmic membrane. For both pathways, an N-terminal signal sequence is required.

The T2SS secretes a collection of degradative enzymes. In *L. pneumophila*, the T2SS is encoded by *lsp* (*Legionella* secretion pathway) and *pilD* genes, which are located in five separated loci, i.e., *lspC, lspDE, lspFGHIJK*, *lspLM*, and *pilD* (Rossier et al. 2004). The *L. pneumophila lsp* and *pilD* mutants are defective for type II secretion and show a growth defect in the amoebal host. As they also display attenuated growth in macrophages, a special role for type II secreted effectors in bacterial pathogenesis was suggested (Hales and Shuman 1999a; Rossier and Cianciotto 2001; Rossier et al. 2004). So far, *Legionella* is the only known intracellular pathogen that encodes a type II secretion system with a role in virulence.

Numerous type II translocated enzymatic activities like lipase, chitinase, esterase, phospholipase A (PLA), ribonuclease, phosphotase and protease activity have been identified (Aragon et al. 2000; Rossier and Cianciotto 2001). The major secreted protein is ProA, a zinc-metalloprotease encoded by *proA/mspA* (Dreyfus and Iglewski 1986; Szeto and Shuman 1990). This protein possesses haemolytic and cytotoxic activities *in vitro* and facilitates tissue damage *in vivo* (Moffat et al. 1994;

Quinn and Tompkins 1989). In addition, ProA promotes the activation of PlaC and a few Lsp secreted proteins and cleaves wide range of substrates including collagen, gelatine and casein (Quinn et al. 1989; Quinn and Tompkins 1989). ProA also degrades or inactivates host cell proteins including human α-1-antitrypsin, TNF- α, IL-12, and CD4 (Conlan et al. 1988; Hell et al. 1993; Mintz et al. 1993). Notably, purified ProA inhibits formation of superoxid anions by monocytes and the activity of natural killer cells (Baskerville et al. 1986; Conlan et al. 1986; Rechnitzer et al. 1989). Other type II secreted proteins include: Map (major acid phosphatase), PlcA (protein with phospholipase C-like activity) and the lipases LipA and LipB (Aragon et al. 2000; Aragon et al. 2002; Rossier and Cianciotto 2001). However, none of these enzymes are separately required for optimal intracellular infection. Further identification and characterization of Type II secretion system substrates is necessary for better understanding of *L. pneumophila* pathogenicity.

3.1.5.2 Type IV secretion systems

The type IV secretion systems (T4SS) are widespread among different bacterial pathogens, such as *Agrobacterium tumefaciens*, *Helicobacter pylori*, *Bordetella pertussis*, *Brucella* sp., *Coxiella burnetii* and others. Bacteria use this machinery in order to deliver macromolecules, even across kingdom barriers (Christie and Vogel 2000). The T4SS are divided into two subgroups. Systems that resemble the *A. tumefaciens* Vir system and the pKM101 Tra system are named type -IVA. Systems that resemble the *L. pneumophila* Dot/Icm system and the IncI plasmids Tra/Trb system (such as R64) are named type -IVB.

Recently a new conjugation system was identified in *L. pneumophila* Corby. It belongs to the type IVA SS and was termed Trb/Tra secretion system. Two similar versions, localized on two different genomic islands (Trb-1 and Trb-2) are present. Trb-1 and Trb-2 are integrated within $tRNA^{Pro}$ gene and tmRNA gene, respectively. At least Trb-1 can be horizontally transferred to other *Legionella* strains by conjugation. Interestingly, a similar locus was found in different non-*pneumophila* species (Glockner et al. 2007).

The Dot/Icm system belongs to the type IVB SS. It was first the identified secretion system responsible for *Legionella* virulence. Using spontaneous and transposon-induced mutants a gene locus, that enables *L. pneumophila* to grow within and to kill human macrophages, was identified (Berger and Isberg 1993; Marra et al. 1992). The *icm* (intracellular replication) and *dot* (defective in organelle trafficking) mutants lost the ability to grow within phagocytes, block phagolysosome fusion, and were also unable to cause disease in animals. In addition, a number of phenotypes have been described to be associated with a functional Dot/Icm apparatus. These include the ability to transfer the RSF1010 plasmid, sensitivity to salt, a contact-dependent cytotoxicity and motility.

The complete *dot/icm* locus comprises a total of 25 *dot/icm* genes in two unlinked chromosomal clusters of approximately 20 kb each and may constitute a single pathogenicity island (Vogel and Isberg 1999). Region I contains 7 genes (*icmV, W,* and *X*, and *dotA, B, C*, and *D*). Region II

contains 18 genes (*icmT, S, R, Q, P, O, N, M, L, K, E, G, C, D, J, B, F*, and *H*). This secretion system functions either before or shortly after bacterial uptake to alter the endocytic pathway in macrophages (Roy et al. 1998; Wiater et al. 1998).

Several effector proteins delivered to the host by the Dot/Icm type IV secretion system were identified. The first effector is RalF, which disrupts vesicle trafficking (Nagai et al. 2002). RalF functions as an exchange factor for the ADP ribosylation factor (ARF) family of guanosine triphosphatases (GTPases), which regulates transport of vesicles between the ER and Golgi. RalF associates with *Legionella*-containing phagosomes within 30 min of uptake in macrophages (Derre and Isberg 2004; Kagan and Roy 2002; Kagan et al. 2004). Other effectors, shown to be secreted by T4SS include LepA and LepB, which are large polypeptides containing a coiled-coil region with weak homology to SNAREs (derived from "soluble NSF attachment receptor). LepA and LepB participate in the trafficking of the *Legionella* phagosome, by playing a role in non-lytic release of *Legionella* from protozoa (Chen et al. 2004). Strains lacking these proteins remain in a replicative vacuole and do not initiate a new round of infection.

LidA is involved in the establishment of the replicative vacuole by recruiting secretory vesicles during the biogenesis of the *Legionella*-containing phagosome (Conover et al. 2003; Derre and Isberg 2005). A recent work shows that LidA binds to a protein that is directly involved in movement of vesicles from the ER to the Golgi. Nevertheless, the biochemical activity of LidA is still unknown.

Combining a yeast two-hybrid approach for initial screening and Cre/loxP-based protein translocation assay, eleven proteins potentially translocated by the Dot/icm were identified (Karimova et al. 1998; Luo and Isberg 2004). They were designated as SidA-G (substrate of Dot/Icm transporter). Most of these effectors have no significant effect on inhibition of phagolysosome maturation or intracellular growth. The single deletion mutants of *sidA*, *sidD*, *sidF* or sidG are not attenuated in virulence of *L. pneumophila* Philadelphia, whereas a quadruple mutant strain, lacking *sidB* and its three paralogs (*sdgABC*), is defective for intracellular growth. SidJ is also a substrate of the Dot/Icm system. Despite its significant intracellular growth defect, the *sidJ* mutant is able to effectively evade fusion with lysosomes. Importantly, recruitment of ER proteins by vacuoles containing the *sidJ* mutant was considerably delayed in both mammalian and amoeba cells. SidJ modulates host cellular pathways, contributing to the trafficking or retention of ER-derived vesicles to *L. pneumophila* vacuoles (Liu, Luo 2007).

Another Dot/Icm substrate is the recently identified SdhA. Macrophages harboring the *sdhA* mutant strain show a severe growth defect accompanied by drastic morphological changes, such as increased nuclear degradation, mitochondrial disruption, membrane permeability, and caspase activation. All together this indicates a role for SdhA in preventing host cell death and demonstrates that *L. pneumophila* is able to manipulate the apoptotic status of infected cells at

different levels, ranging from control of major pathways to inhibition of specific pro-death proteins (Laguna et al. 2006).

Other effector molecules secreted by Dot/Icm and interfering with membrane trafficking were identified by an intelligent screening method based on yeast (Shohdy et al. 2005). The identified proteins were designated vacuole protein sorting inhibitor proteins (Vips). VipA is a coiled-coil protein, VipD is a patatin domain-containing protein and VipF contains an acetyltransferase domain. Processing studies in yeast indicate that VipA, VipD, and VipF inhibit lysosomal protein trafficking by different mechanisms. Overexpressing VipA influences carboxypeptidase Y trafficking, whereas VipD interferes with multivesicular body formation in late endosomes and with ER-to-golgi body transport.

A comprehensive analysis of identity and function of effectors translocated by the Dot/icm apparatus is crucial in determination of how this bacterium establishes a replication vacuole. It is reported that proteins translocated from bacteria to host cells can also be transferred between bacterial cells. Interestingly, many effector proteins show no sequence similarity to proteins from other characterized organisms. This may indicate the uniqueness of mechanisms which serve *L. pneumophila* to subvert eukaryotic cell functions (Bruggemann et al. 2006).

3.1.5.3 Type V secretion proteins

The type V secretion pathway, also called the autotransporter system uses the sec protein-translocation system for crossing the inner membrane. Proteins secreted via this pathway have similarities in their primary structures as well as striking similarities in their modes of biogenesis. *L. pneumophila* strain Paris encodes a predicted autotransporter protein, which has the typical features of type V secretion proteins. There is a N-terminal leader peptide for secretion across the inner membrane and a C-terminal domain that forms a pore in the outer membrane through which the passenger domain passes to the cell surface (Cazalet et al. 2004). So far, the protein with similarity to type V secretion proteins was detected in only one *L. pneumophila* strain.

3.1.5.4 Additional virulence factors

Besides the secretion systems and their effectors *Legionella* possess numerous virulence factors. These factors participate in adherence, hydrogen peroxide decomposition, amino acid biosynthesis and transport. The major outer membrane protein (MOMP) is an important member of the group of factors involved in adherence and entry into the host cells. It is a porin shown to mediate phagocytosis by binding to the monocyte receptors CR1 and CR3 (Bellinger-Kawahara and Horwitz 1990; Gabay et al. 1985; Payne and Horwitz 1987). Mip (macrophage infectivity potentiator) and the cell-associated toxin RtxA (repeats in structural toxin) belong to the same group of proteins. Mip exhibits collagen binding activity which has a function in penetration of the lung epithelial barrier during infection (Wagner et al. 2007). RtxA is a cytotoxic protein which

belongs to a large family of pore-forming cytolysins present in many different bacterial pathogens (Cianciotto et al. 1989; Cianciotto and Fields 1992; Cirillo et al. 2002; Fischer et al. 1992; Wagner et al. 2007; Welch 1991).

Bacterial phospholipases (PlaC, PlaB) are also involved in a number of disease-promoting actions. They are able to hydrolyse phospholipids to bioactive molecules, which influence host signalling pathways and form pores in lipid layers (Flieger et al. 2000).

Consistent with the observation of M. Swanson that the transmissive phenotype (associated with cytotoxicity) is induced when *Legionella* enter into the stationary phase, an additional group of virulence factors can be designated (Molofsky and Swanson 2004; Swanson and Hammer 2000). The ppGpp alarmone synthesized by RelA in response to amino acid starvation, has been proposed to activate two parallel signalling pathways in *L. pneumophila*, one of which is controlled by RpoS while the other is controlled by the LetA/S two-component regulatory system (Bachman and Swanson 2004; Hales and Shuman 1999b). These two pathways coordinate induction of several transmissive phase virulence traits. Proteins induced in the stationary phase often promote virulence, as the flagellum protein FlaA is essential for invasion to host cells, and catalase-peroxidase enzymes KatA and KatB catalyze the decomposition of hydrogen peroxide. These growth stage-dependent factors may thus promote intracellular survival and growth of *L. pneumophila* (Bandyopadhyay et al. 2003; Bandyopadhyay et al. 2004; Dietrich et al. 2001; Heuner and Steinert 2003).

Genome analysis of several pathogenic *Legionella* strains revealed a cluster of so-called eukaryotic-like proteins. These genes encode proteins with a high similarity to eukaryotic proteins or motifs known to be involved in protein-protein interaction, such as ankyrin repeats, F-box, U-box and Sel-1(tetratrico peptide repeat) domains (Bruggemann et al. 2006). Elucidation of the role of ankyrin repeats containing proteins appears to be complicated because they are involved in many cellular processes. Nevertheless, they were predicted to be involved in the interaction with host cytoskeleton or in targeting proteins to ER or plasma membrane. Since F-box and U-box proteins participate in the modulation of eukaryotic ubiquitination machinery, presence of these proteins in *Legionella* genome suggests that the pathogen might use general proteasome processes to its advantage (Angot et al. 2007). Other eukaryotic-like proteins encoded in the genome (sphingosine-1-phosphate lyase and secreted apyrases) may potentially interfere with the autophagy pathway. Three eukaryotic-like serine/threonin protein kinases are thought to be involved in the autophagy pathway as well. They inhibit the phagolysosome fusion, promote intracellular survival and interfere with host signal transduction pathways in the case of other intracellular pathogens (Barz et al. 2000; Walburger et al. 2004).

3.1.6 Host factors implicated in *Legionella* pathogenicity

In spite of similar mechanisms of host cell internalisation of *Legionella* a number of differences between the host systems can be designated. The host and bacterial factors implicated in pathogenicity during *Legionella* infection are summarised in Table 3-1.

Table 3-1 Molecular determinants of *L. pneumophila* and eukaryotic cells relevant for host-pathogen interaction during intracellular bacterial life cycle (modified (Hilbi et al. 2007)

	Mammalian cells[a]	Dictyostelium	Amoeba[b]	Drosophila[c]
***Legionella* factors**	Icm/Dot T4SS: RalF, LidA, SidM SidC Mip, PmiA FliA (δ^{28}) LetA/GacA Type IV pili Tat (TatBC) LidA, SidM ProA MOMP RtxA	Icm/Dot T4SS LepA, LepB, SidC Mip FliA (δ^{28})	Icm/Dot T4SS SidC Mip, PmiA, RelA FliA (δ^{28}), RpoS(δ^{38}) LetA/GacA Type IV pili Tat (TatBC) Lsp T2SS LipidA Vips	Icm/Dot SS
Host factors	Nramp1 PI(4)P Arf1, Sar1, Rab1 Sec22+TRAPP Cdc48/p97 (ERAD) Proteasome	Nramp1 PI(3)P; PI(4)P G protein (β subunit) Coronin Myosin I, actin, other cytoskeletal proteins RtoA	Lectin (Gal/GalNac)	Arf1, Sar1, Rabfc Sec22+TRAPP Cdc48/p97(ERAD) Proteasome

a. Primary macrophages (murine bone marrow-derived, human monocyte-derived), macrophage-like cell lines (murine RAW264.7, human HL-60, human U937, human MonoMac6), epithelial cells (HeLa), fibroblasts (human embryonic kidney), or mouse strains.
b. *Acanthamoeba castellanii, Acanthamoeba polyphaga, Hartmanella vermiformis or Tetrahymena pyriformis*
c. *Drosophila melanogaster* Kc167 or S2 cells (macrophage-like phagocytes), or adult flies.

The soil living amoeba *D. discoideum* appears to be a valuable system for studing of *Legionella*-host interaction. Advantages of this organism are the haploid and small size of genome, and availability of mutants and cellular markers. All factors together make *D. discoideum* an attractive system for detailed characterization of *Legionella* intracellular cycle.

3.2 *D. discoideum*

3.2.1 General characteristics of *D. discoideum*

D. discoideum is a cellular slime mold which is a comparatively simple unicellular eukaryote. Since discovery by Raper in 1935, it is a popular and productive model system for studying the molecular basis of cell and developmental biology (Raper and Smith 1939). *D. discoideum* grows by mitotic division of single cells that feed by phagocytosis on bacteria or by macropinocytosis on simple axenic liquid medium. Growth in axenic medium makes it possible to cultivate *D. discoideum* under laboratory conditions and simplify a variety of genetic manipulations. The term "social amoebae", applicable to *D. discoideum,* derives from the behaviour of the cells. Upon starvation about $\approx 10^5$ cells aggregate by chemotaxis and forme the multi-cellular aggregate with a fruiting body, consisting of 20 % dead stalk cells and 80 % spore cells. The stalk supporting one or more balls of spores, which consist of cells protected by resistant cell walls, and become new amoebae once food is available. *D. discoideum* also undergoes sexual development. Under dark and submerged conditions, the amoeboid cells become sexually mature, fuse with mating-type cells and become zygotes. The zygotes then secrete cAMP to gather the surrounding cells, engulf the cells as nutrients, and develop into dormant structures called macrocysts (Erdos et al. 1976; O'Day and Durston 1979).

The genetic information of *D. discoideum* is carried on six chromosomes with sizes ranging from 4 to 7 Mb, resulting in a total of about 34 Mb of DNA, a multicopy 90 kb extrachromosomal element that harbors the rRNA genes, and the 55 kb mitochondrial genome. The genome encodes about 10300 proteins, including protein families involved in cytoskeleton control, post-translation protein modification, detoxification, secondary metabolism, cell adhesion and signal transduction. Moreover, a large number of proteins closely related to their mammalian orthologs, including 33 *D. discoideum* proteins have a high homology to human disease-related proteins (Eichinger et al. 2005).

Most researchers are currently using the axenically growing strain Ax-1, which was first isolated by continuous subculturing of DdB (NC-4) derivate colonies that showed more synchronous development and less spreading colony morphology (Sussman and Sussman 1967). Later, a strain that was able to grow on simplified medium was isolated by prolonged subculturing of Ax-1 and designated as Ax-2 (AX2) (Watts and Ashworth 1970). Nearly at the same time, the AX3 strain was isolated independently by using N-methyl- N'-nitro N-nitrosoguanidine, and grows as well as AX2 strain in the defined HL5 medium (Franke and Kessin 1977; Loomis 1971).

3.2.2 *D. discoideum* as model organism

Many features of *D. discoideum* make it a very attractive model system for cellular biologists. First

of all, this organism has a haploid nature that allows us to generate a variety of mutants and simplify verification of their phenotype. At the moment several molecular genetic tools are available, including the ability to perform homologous gene replacements, random insertional mutagenesis (termed restriction-enzyme-mediated integration, REMI), multiple-gene deletions and RNA interference (RNAi) technologies (Chen et al. 1994; De Lozanne and Spudich 1987; Faix et al. 2004; Kuhlmann et al. 2006; Kuspa and Loomis 1992). Second, the easy cultivation and short multiplication time facilitate generation of multiple cell markers and progress in study of cell signalling pathways. Third, the *D. discoideum* is an efficient phagocyte, which shares its chemotactic capacity with leukocytes, and the process of particle uptake appears to be similar to macrophage phagocytosis (Noegel and Schleicher 2000; Rupper and Cardelli 2001; Steinert and Heuner 2005).

D. discoideum is a useful organism not only for the study of different cellular processes, but also for the investigation of bacterial virulence and the implication of the host side in infection. As such *D. discoideum* has been successfully used as a model for infection with several pathogens, like *Pseudomonas aerogenosa* (Pukatzki et al. 2002), *Vibrio cholerae* (Pukatzki et al. 2006) and *Mycobacterium* species (Pukatzki et al. 2002; Skriwan et al. 2002).

Previously a number of different assays utilising *D. discoideum* as a host model organism were established. For example, the screening for resistance to *D. discoideum* predation might reveal virulence factors produced by pathogenic strains. Therefore, *D. discoideum* can be useful in searching for new determinants implicated in pathogenicity in a mutagenized pathogen library. Phagocytosis assays with mutated *D. discoideum* host cells or mutated pathogens such as *L. pneumophila*, *Mycobacterium spp*. and *Cryptococcus neoformans* can reveal important aspects of the initial steps of infection. Furthemore, the use of specific cellular inhibitors can help to identify the involved signal transduction pathways, and infection assays in which the intracellular growth of organism is analysed can illuminate the outcome of the host-pathogen interaction (Unal and Steinert 2006).

3.2.3 Phagocytosis

3.2.3.1 General aspects of phagosome biogenesis and composition

Phagocytosis is a conserved cellular process and plays a fundamental role in the immune system for the defence against microbes and the clearing of apoptotic cells in eukaryotes. The kinetics and regulation of phagosomal maturation in *D. discoideum* are comparable to phagosomal maturation in mammalian phagocytes. Phagocytosis is a multi-step process, involving the recognition of particulate material, their F-actin-driven engulfment and subsequent destruction (Desjardins 2003; Muller-Taubenberger et al. 2001). When compared to unicellular organisms, mammalian professional phagocytes, such as neutrophils, dendritic cells, and macrophages, display a more

varied set of phagocytic receptors, coupled to distinct signal transduction pathways. In mammalian phagocytes, the study of phagocytic pathways has been focused mainly on those triggered by the Fc receptors and complement receptors, which mediate phagocytosis of antibody-opsonized and complement-opsonized particles, respectively (Swanson and Hoppe 2004). Although it is still difficult to define how the signalling network is orchestrated during phagocytosis, numerous studies demonstrate the importance of small GTP-binding proteins, the cytoskeleton, phosphoinositide metabolism and pH regulation in this process (Duhon and Cardelli 2002; Hart and Young 1991; Rupper and Cardelli 2001; Vieira et al. 2001).

The small GTPases are a family of proteins that bind to guanosine triphosphate (GTP) and regulate a wide variety of processes in the cell, including signal transduction, translation and transport of vesicles within the cell. The group of small GTP-binding proteins (Rap1, Arf6, Rho and Rab) control and coordinate the successive steps of the phagocytic process. Moreover, these molecules are often targeted by microbial virulence factors. Once particles have bound to host cells, Rho proteins control the remodelling of the actin cytoskeleton that drives uptake. Which particular signalling pathway and Rho protein is activated during uptake will depend, however, on the nature of the initial receptor/ligand interaction at the cell surface. Generally a particle binds and triggers the local recruitment and activation of one or several Rho proteins. This mediates the nucleation and the dendritic assembly of actin filaments and enrichment of this protein about the nascent phagosome in an Arp2/3-dependent manner (Pollard and Borisy 2003). The delivery of endosome-derived membranes to a forming phagosome is also required for optimal phagocytosis. This process is controlled by Arf-family member Arf 6 and by Rab proteins. The GTPase-controlled pathways leading to membrane delivery and actin polymerisation are independently activated upon particle binding. The small GTP-binding proteins play a crucial role not only in phagocytic uptake, but also in several responses associated with phagocytosis, such as the activation of the NADPH oxidase and phagosome maturation (Abo et al. 1991; Knaus et al. 1991). The Rab7 GTPase in *D. discoideum* has also been shown to regulate delivery of lysosomal enzymes from the endosomal compartment (Buczynski et al. 1997).

Maturing phagosomes are able to move along microtubules inside the cell (Blocker et al. 1997). This function may be controlled by an effector of Rab7 termed RILP, which binds to dynein-dynactin, and has a role in the attachment of phagosomes to microtubules (Cantalupo et al. 2001; Harrison et al. 2003). Additionally, this process occurs via Arp2/3 in *D. discoideum* cells. The Arp2/3 complex is recruited to the PM (plasma membrane) in the place of mechanical pressure of phagosome and initiates the actin-mediated phagosomal rocketing (Clarke and Maddera 2006b).

Phagosome maturation is also accompanied by the acquisition of sets of lipid raft-associated proteins, including Flotillin-1 and subunits of the proton pump ATPases. Proton pump ATPases appear to be absent in nascent phagosomes and to be recruited from endocytic compartments during phagosome maturation (Dermine et al. 2001). Lipid microdomains on phagosomes may

serve as platforms for the assembly and nucleation of actin (Defacque et al. 2002). Interestingly, the lipid composition of phagosomes is modified during maturation. While early phagosomes are preferentially enriched with phosphatidylcholine, late phagosome are enriched with sphingomyelin (Desjardins et al. 1994).

Recently, several organelles including recycling endosomes, late endosomes, lysosomes and ER were shown to display the ability to fuse with the PM and provide membrane source for phagosome formation (Desjardins 2003). A number of contradictory studies about presence and evidence of ER in phagocytosis were published. For example, in neutrophils the entry of both latex beads and *Leishmania* parasites does not recruit ER, while *L. pneumophila* and *Brucella abortus* were shown to be present in phagosomes having ER features (Desjardins et al. 1994; Horwitz 1983; Pizarro-Cerda et al. 1998; Swanson and Isberg 1995; Tilney et al. 2001).

Recently it was shown that the SNARE molecule Sec22b is involved in the regulation of membrane fusion between the ER and PM during phagocytosis. However, while Sec22b is required for the phagocytosis of large particles (> 3 μm), it is not essential for the internalization of smaller beads (< 1 μm). This suggests the contribution of ER to phagocytosis might vary according to the type of particle internalized (Becker et al. 2005). Interestingly, the Gerisch group showed that the ER is likely to play a direct role in the formation of phagosomes in *D. discoideum* cells. They showed that a double knockout mutant of the ER proteins calnexin and calreticulin was abolished in phagocytosis (Muller-Taubenberger et al. 2001).

The machinery for ubiquitination and proteosomal degradation was also detected in early phagosomes (Houde et al. 2003). Degradative peptides enter the classical ER-Golgi-PM pathway or phagosomes through the TAP (Transporter Associated with antigen Processing). The contribution of the ER to phagosomes provides sets of transporters: Sec61 translocon, the putative channel formed by the recently identified Derlin-1 or chaperones involved in retro-translocation (calnexin and GRP78/Bip) (Desjardins et al. 2005; Jutras and Desjardins 2005).

Modulation of phagosomal function may also involve phosphoinositide metabolism, which regulates the level of inositol and phosphatidylinositol phosphate in the membrane and plays a pivotal role in the regulation of signal transduction, actin remodelling and membrane dynamics during phagocytosis. The role of different types of phosphatidylinositol kinases in phagocytosis in general and in intracellular survival of the pathogens modulating phagocytosis of host cells was recently reviewed by Hilbi (Hilbi 2006).

3.2.3.2 *D. discoideum* phagosome composition

Legionella enters its host cells by phagocytosis. Accordingly, the characterization of the phagosomal composition facilitates the understanding of the underlying mechanisms. The recently published composition of *D. discoideum* latex beads phagosome includes not only the expected

lysosomal proteins but also a large variety of proteins involved in regulation of membrane trafficking, components of signal transduction and the cytoskeleton, as well as a subset of heterotrimeric G protein subunits involved in signal transduction. In addition, factors involved in protein biosynthesis, like ribosomal components, tRNA synthetases and translation elongation factors (which are actin-binding proteins) were detected. This can be explained either by protein synthesis taking place on the phagosome itself or by substantial association/fusion of phagosomes with the ER. Moreover, the temporal profiles of phagosome proteins during maturation were generated. In particular, based on the presence of Gα2 and Gα4 protein spots at the early time point on protein 2-D gels it was suggested that they may be involved in phagocytic uptake (Gotthardt et al. 2006a).

3.2.4 *D. discoideum* determinants of *Legionella* infection

Legionella was the first environmental pathogen shown to replicate within *D. discoideum* (Hagele et al. 2000; Solomon and Isberg 2000; Solomon et al. 2000). Since then the body of knowledge about the interaction of these organisms has been growing. *D. discoideum* mutants were used for determination of genetic host factors responsible for susceptibility and resistance to infections. A number of green fluorescence-marked proteins were analyzed for a role in phagocytosis and intracellular growth of *L. pneumophila*. In addition, a *D. discoideum* microarray was utilized for analysis of transcriptional changes that occur during *Legionella* infection.

3.2.4.1 Analysis of *D. discoideum* factors implicated in *Legionella* infection

Many *D. discoideum* mutants were analyzed for a role in phagocytosis and intracellular growth of *L. pneumophila*. The G-protein β subunit mutant, which is completely defective in heterotrimeric G-protein signalling in *D. discoideum*, allows slightly less growth of *L. pneumophila* than its parental strain. The absence of α-actinin, LimC/D, or villidin also has this effect (Fajardo et al. 2004). At the same time, mutants with impaired actin cytoskeleton function (coronin, profiling, comitin and the double myosin I mutant, myoA/B) were better at supporting *L. pneumophila* growth than their parental strains (Hagele et al. 2000; Skriwan et al. 2002; Solomon et al. 2000). A more severe defect was caused by the absence of RtoA, a protein involved in vesicle trafficking and essential for the modification and expansion of the *L. pneumophila*-containing compartment (Li et al. 2005). The *D. discoideum rto*A(-) strain was shown to be marginally defective in uptake of *Legionella*.

Intracellular replication of *L. pneumophila* was found to be enhanced in mutants lacking the metal cation transporter Nramp1 (natural resistance-associated membrane protein-1), and in the case of *L. pneumophila*, blocked by overexpression of the corresponding gene (Peracino et al. 2006).

An analysis of the role of phosphoinositide metabolism in *L. pneumophila*-infected *D. discoideum* revealed that deletion or pharmacological inhibition of PI(3)K is not required for uptake of wild-

type *L. pneumophila*, but promotes intracellular replication and impairs the transition from tight to spacious *Legionella* vacuole (Hilbi 2006). While phosphatidylinositol-3 phosphate (PI(3)K) inhibitors apparently have no effect on intracellular replication of *L. pneumophila* within macrophages, phosphatidylinositol-4 phosphate (PI(4)P) was found to accumulate in *D. discoideum* phagosomes in an Dot/Icm-dependent manner and was also present on macrophage phagosomes (Molmeret et al. 2004; Weber et al. 2006).

3.2.4.2 Transcriptional changes during *Legionella* infection

The transcriptional changes on *D. discoideum* during *Legionella* infection were examined with the help of a *D. discoideum* DNA microarray (Farbrother et al. 2006). The array consisted of cDNA covering approximately half of the *D. discoideum* genome. The differentially expressed genes were thought to be either required for successful *Legionella* infection and proliferation, or to be involved in host-specific defence mechanisms in response to infection. The largest number of differentially transcribed genes was identified at 24 hours post infection, at which time a set of 131 genes was regulated in a *Legionella*-specific manner. Similarly regulated genes were then clustered for further detailed analysis, which indicated enrichments of genes involved in translation, proteolysis, stress response and a set of lipid modifying enzymes. Interestingly, expression of the *rtoA* gene, which promotes intracellular replication of *L. pneumophila* in *D. discoideum*, was two fold up-regulated at 24 hours post infection.

3.3 Aim of the study

The interactions between host and pathogen during *Legionella* infection are very complex. The major mechanism of intracellular survival and replication of the pathogen is the ability to reprogram the phagosome maturation. However, the molecular mechanism of this process is unknown.

Since social amoeba *D. discoideum* has proven to be a representative host model system for analysis of intracellular aspects of *Legionella* pathogenicity we have used this organism in the present work. The goal of this study is to gain a better insight into the host response during this process. The work here has several aims:

- Establishing of new screening methods for analysis of *Legionella* virulence and host-pathogen interaction using *D. discoideum* as a model system.
- Establishing an efficient protocol for isolation of *Legionella*-containing phagosomes from infected *D. discoideum* cells with high purity. The method of isolation should be compatible with subsequent mass spectrometry analysis of membrane proteins.
- Identification and functional analysis of phagosomal proteins.
- Analysis of phagosomal proteome alteration between *Legionella* species with different intracellular phenotypes and during infection.

4 Material and methods

4.1 Material

4.1.1 Equipment

The equipment used in this study is listed in Table 4-1.

Table 4-1 List of used devices

Device	Manufacture
Analysis scales	Chyo JL 180
Autoclave	Fedegari FOM/B50
	Fedegari Teknomara 9191E
Cleanbench	Nunc Microflow 50726
Chemi Lux-Imager	Intas Science, GmbH, Göttingen
Dura-Grind Stainless Steel Dounce Tissue Grinders	Wheaton Science
Icemaker	Scotsman AF 20
Electromagnet	Scotsman AF 20
Electron Microscope	Zeiss EM10
Electrophoresis apparatus for SDS-PAGE	Pharmacia, BioRad
Electroporation-apparatus	Pharmacia, BioRad
Electrotransfer- apparatus	Mini Trans-Blot Cell, BioRad
Ettan IPGphor Isoelectric Focusing Unit	Amersham Biosciences
Ettan Spot Picker	Amersham Biosciences
Gel documentation apparat	Gel Doc 2000, Bio Rad
Incubators	Heraeus B5050E, Memmert Tv40b
Magnetic stirrer	Sorvall RT 600
Microwave	Moulinex
OctoMACS™ Separation Unit	Miltenyi Biotec
PCR-Thermocycler	Biometra, T3 Thermocycler
pH Meter	Metrohm-Herisau E512
Photometer	Amersham Pharmacia Biotech
	Ultrospec 3000
Pipettes	Gilson, Eppendorff
Refrigerator box -20ºC	Privileg Senator
Refrigerator box -80ºC	REVCO
Rehydration tray	Amersham Biosciences
Sonicator	Bandelin Sonoplus HD70, Tip UW70

Speedvac	Savant SC110
Sterile bank	Nunc Microflow 50726
Shaker	Innova TM 4300, Bühler
Table centrifuge	Eppendorf Zentrifuge 5415C
Thermoblock	Eppendorf Thermostat 5320
Thermostat	Heraeus B5050E, Mammert Tv40b
Transilluminator	Appligene, Frankreich
Thyphoon 8600	Amersham Biosciences
Vortex	GLW
UV-Crosslinker	BioRad

4.1.2 Other Materials

Plastic and related articles were purchased from the following companies: Nunc (Roskilde), DK; Sartorius (Göttingen), Falco/Becton Dickinson (Heidelberg), B. Braun (Melsungen), Eppendorf (Hamburg) Greiner, Nürtingen and Schleicher & Schüll (Dassel). Nylon membranes (Zeta Probe GT) were purchased from Biorad and Nitrocellulose membranes (Whatman-paper) from Schleicher & Schüll.

4.1.3 Chemicals and Enzymes

All chemicals and antibiotics were supplied by Amersham Biosciences (München), Applied Biosystems (Foster City; USA), Biochrom (Berlin), Biolabs (Frankfurt am Main), Difco (Augsburg), Dianova (Hamburg), Gibco BRL (Eggenstein), Invitrogen (Karlsruhe), MBI Fermentas GmbH (St.Leon-Rot), Merck (Darmstadt), Oxoid (Wesel), Promega (Mannheim), QIAGEN (Hilden), Roche Diagnostic (Mannheim), Roth (Karlsruhe), Sigma (Deisenhofen), Sigma-Aldrich GmbH (München). Enzymes were obtained from MBI Fermentas (St. Leon-Roth), Roche (Mannheim), and Gibco (Eggenstein).

4.1.4 Bacterial Strains and Cells

The bacterial and eukaryotic strains used in this study are listed in Table 4-2.

Table 4-2 List of used bacterial and eukaryotic strains.

Strain	Medium and growth condition	Reference, source
E. coli K12 DH5α	LB, 37 ºC	Bethesda, Research
Klebsiella aerogenes	SM, 37 ºC	M. Schleicher, LMU München
L. pneumophila Corby, Sg.1	BCYE, 37 ºC	Jepras et al., 1985
L. pneumophila 18398/98 patient isolate	BCYE, 37 ºC	Lück, Dresden
L. pneumophila Philadelphia I, Sg.1	BCYE, 37 ºC	ATCC 33152
L. hackeliae, Sg.1	BCYE, 37 ºC	ATCC 33250
L. micdadei	BCYE, 37 ºC	ATCC 33218
L. micdadei L01-500, patient isolate	BCYE, 37 ºC	Lück, Dresden
L. micdadei W02-539, eviroment isolate	BCYE, 37 ºC	Lück, Dresden
L. bozemanii	BCYE, 37 ºC	ATCC 33217
L. erythra	BCYE, 37 ºC	P. Fettes, Ulm University
L. lytica	BCYE, 30 ºC	Prosansky, Lublin
L. longbeachae Sg.1	BCYE, 37 ºC	ATCC 33462
LLAP10	BCYE, 30 ºC	CDC, Atlanta
D. discoideum discoideum AX2	HL5, 24 ºC	M. Schleicher, LMU München

4.1.5 Antibodies, plasmids, and markers

The antibodies used in this study are listed in Table 4-3.

Table 4-3 List of antibodies used in this study

Antibody description	Dilution	Reference, source
Mouse-monoclonal α-mitoporin-antibody (*D.d.*) 70-100-1	1:50	Annette Müller-Taubenberger
Mouse- monoclonal calnexin antibody (*D. d.*) 270-390-2	1:50	Annette Müller-Taubenberger
Mouse- monoclonal vatA antibody (*D.d.*) 221-35-2	1:50	Annette Müller-Taubenberger
Rabbit CAD-1 IgG (*D.d.*)	1:2000	Prof. Chi-Hung Siu
Maus-monoclonal actin-1 antibody (*D.d.*)	1:5	Prof. M. Sleicher
Anti-rabbit DdCAD-1 IgG	1:2000	Prof. Chi-Hung Siu
Second antibody; Polyclonal Goal Anti-Mouse Immunoglobulins/HRP	1:5000	Dianova, Hamburg
Second antibody; Polyclonal Goal Anti-Rabbit Immunoglobulins/HRP	1:2000	Dianova, Hamburg

4.1.6 Vector

For the generation of Transposon mutant library the pCDP05a (Fig.4-1) was used (Pope et al. 1994)

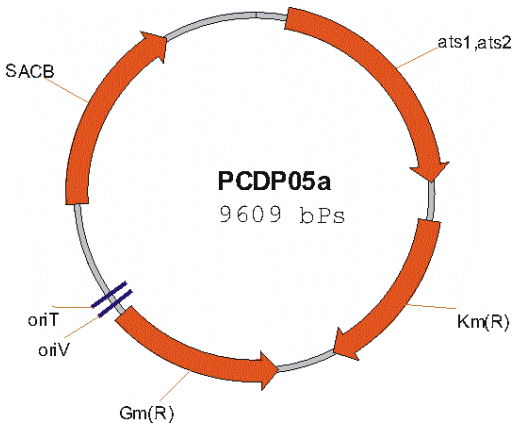


Fig. 4-1 Map of the transposon mutagenesis vector pCDP05a
Genes are designated as follows: kanamycin resistance Km(R), chloramphenicol resistance Cm(R), levansucrase (SACB), double mutant ATS (ats1, ats2). Arrows indicate the direction of gene transcription. The approximate locations of ColE1 origin of replication (oriV) and conjugal origin of transfer (oriT) are also noted.

4.1.7 Oligonucleotides

The primers used in this study are listed in Table 4-4.

Table 4-4 List of primers used in this study

Primer	Primer Sequence	Annealing temperature
Tn1	5'-TGA TTT TGA TGA CGA GCG-3'	51.4 ºC
Tn2	5'-GTG ACG ACT GAA TCC GGT-3'	56.0 ºC
Tn3IS	5'-CCT TAA CTT AAT GAT TTT TAC-3'	48.1 ºC
Km2_L	5'- GAT AAT GTC GGG CAA TCA GGT G-3'	55.8 ºC
Km2_R	5'- ACA GGA ATC GAA TGC AAC CG-3'	54.8 ºC

4.1.8 Kits

The following kits were used in this study:

Roti-Nanoguant (Roth), Plasmid Midi Kit, QIAGEN, Hilden, Amersham ECL™ Direct Acid Labelling and Detection System, und ECL™ Advance, CyDye DIGE Flour Labelling Kit for Scarce Samples, Amersham Biosciences, DIG Nucleic Acid Detection Kit Roche diagnostic, Mannheim, Pro-Q Diamond phosphoprotein Gel Stain (Molecular Probes).

4.1.9 Markers (proteins, DNA, phosphoproteins, DIGE marker)

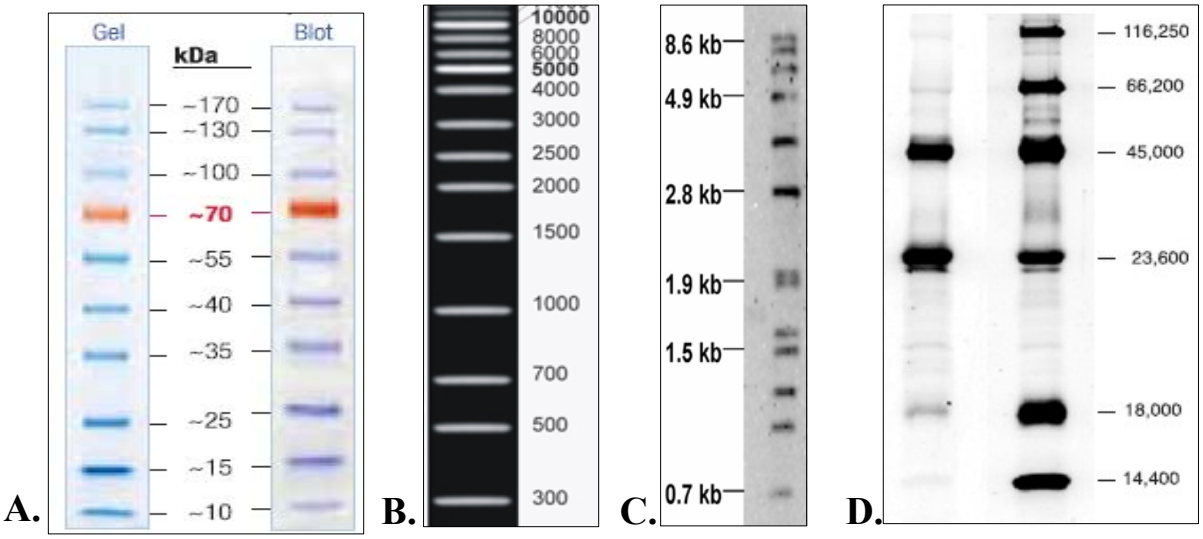


Fig.4-1 The following markers were used in this study
A. PageRuler prestained Protein Ladder (Fermentas)
B. FastRuler DNA Ladder, High Range (Fermentas)
C. DIG-labelled control DNA (Roche Applied Science)
D. Phosphorylated (1 line) and non-phosphorylated (2 line) proteins in PeppermintStick phosphoprotein molecular weight standards (Molecular Probes)

4.1.10 Buffers, solutions and media

The buffers and solutions used in this study are listed in Table 4-5.

Table 4-5 List of buffers and solution used in this study

Name of the buffer	Components	Amount or concentration
10 x PBS (pH 7.2)	KCl K_2HPO_4 Na_2HPO_4 NaCl Ad H_2O_{bidest} Adjust to pH 7.2 with 1N HCl	2 g 2 g 11.5 g 80 g 1000 ml
TE (pH 8.0)	Tris-HCl Na_2EDTA	10 mM 1 mM
50 x Soerensen buffer	K_2HPO_4 Na_2HPO_4 Ad H_2O_{bidest}	99.86 g 17.8 g 1000 ml
50 x TAE (pH 8.0)	Tris Base Acetic Acid Na_2EDTA (0.5M)	2.0 M 1.0 M 0.1 M
10 x SDS PAGE running buffer	Tris Base Glycin SDS Ad H_2O_{bidest}	30.3 g 144.0 g 10,0 g 1000 ml
Towbin buffer (pH 8.3)	Tris Base Glycin Methanol Ad H_2O_{bidest}	3 g 14.4 g 20% 1000 ml
Stripping buffer (pH 7.6)	62.5 mM Tris-HCl 2 % SDS Ad H_2O_{bidest} Adjust to pH 7.6 with HCl Ad 700 µl β- Mercaptoethanol for 100 ml buffer before use.	3.63 g 10 g 500 ml
Protein preparation:		
Cell lysis buffer (pH 8.0)	Urea Thiourea CHAPS Tris Adjust to pH 8.0 with 1.0 M HCl	7 M 2 M 4 % (w/v) 30 mM
1 x Sample buffer	Urea Thiourea CHAPS	7 M 2 M 4 % (w/v)

2 x Sample buffer	Urea	7 M
	Thiourea	2 M
	CHAPS	4 % (w/v)
	Pharmalyte (pH3-10)	2 % (v/v)
	DTT	1 mg/ml
Rehydration buffer	Urea	7 M
	Thiourea	2 M
	CHAPS	4 % (w/v)
	Pharmalyte (pH3-10)	1 % (v/v)
	DTT	1 mg/ml
Equilibration buffer	Urea	6 M
	Tris-HCl (pH 8.0)	0.1 M
	Glycerol	30 %
	SDS	2 %
	DTT	0.5 % (w/v)
HB buffer	Na_2EGTA (0.5M)	0.5 mM
	HEPES	20 mM
	Sucrose	250 mM
	Ad H_2O_{bidest}	1000 ml
DNA isolation:		
TNE buffer	Tris HCl (pH 7.5)	10 mM
	NaCl	10 mM
	EDTA	10 mM
TNEX buffer	Tris HCl (pH 7.5)	10 mM
	NaCl	10 mM
	EDTA	10 mM
	Triton X100	1 %
Southern blot and hybridization:		
Depurination buffer	HCl	250 mM
Denaturation buffer	NaOH	0.5 M
	NaCl	1.5 M
Neutralisation buffer(pH 7.5)	Tris-HCl	0.5 M
	NaCl	1.5 M
20 x SSC (pH 7.0), Autoclaved	NaCl	3 M
	Sodium Citrate	300 mM
Equilibration buffer	5X SSC	--
Buffer for blotting buffer	20X SSC	--

Low stringency buffer	2X SSC SDS	-- 0.1 %
High stringency buffer	0.1X SSC SDS	-- 0.1 %
Washing buffer (pH 7.5)	Maleic Acid NaCl Tween-20	0.1 M 0.15 M 0.3 % (v/v)
Maleic Acid buffer (pH 7.5) adjust with NaOH tablets	Maleic Acid NaCl	0.2 M 0.15 M
Detection buffer (pH 9.5)	Tris NaCl	0.1 M 0.1 M
20 x SSPE buffer (pH 7.4), autoclaved	NaCl NaH_2HPO_4* H_2O EDTA Ad H_2O_{bidest} Adjust to pH 7.4 with 10N NaOH	3 M 200 mM 20 mM
50 x Denhardt s solution	Ficoll Bovine serum albumin (BSA) Polyvinylpyrrolidone	1 % 1 % 1 %

The culture media used in this study are listed in Table 4-6.

Table 4-6 List of culture media used in this study

Eschrichia coli		
Luria-Bertani (LB) Medium	Trypton Yeast powder extract NaCl Ad H_2O_{bidest}	10 g 5 g 5 g 1000 ml
Legionella spp.		
AYE Medium (Aces-buffered Yeast-Extract Medium)	Yeast powder extract ACES Ad H_2O_{bidest} Adjust to pH 6.9 with 10 M KOH Suspend and autoclave. Let cool to ~50 ºC and add filter sterilized: L- Cystein $FeNO_3$	10 g 10 g 1000 ml 0.4 g 0.25 g

BCYE agar	Yeast powder extract	10 g
	ACES	10 g
	Ad H_2Obidest	900 ml
	Adjust to pH 6.9 with 10 M KOH	
	Activated charcoal	2 g
	Agar	15 g
	Suspend and autoclave. Let cool to ~50 ºC and add filter sterilized:	
	Cystein in 10 ml	0.4 g
	$FeNO_3$ in 10 ml H_2O_{bidest}	0.25 g
YEB+-Medium (Yeast-Extract Broth)	Yeast powder extract	1 g
	Ad H_2O_{bidest}	100 ml
	Suspend and autoclave. Let cool and add *Legionella* grow Supplement (Oxiod SR0110A)	
D. discoideum		
HL5 medium	Proteose peptone	14.3 g
	Yeast powder extract	7.15 g
	Na_2HPO_4	1.28 g
	K_2HPO_4	0.49 g
	Ad H_2O_{bidest}	900 ml
	Adjust to pH 7.5 with 10 M KOH	
	Glucose	15.4 g
	Ad H_2O_{bidest}	100 ml
	Suspend all components except glucose in H_2O_{bidest} and autoclave. Then add filter sterilized glucose solution.	
Infection medium	HL5 and 1x Soerensen buffer mix in ratio 1:1	
SM agar	Bacto peptone(Difco)	10 g
	Yeast powder extract	1 g
	$MgSO_4$	1 g
	KH_2PO_4	2.2 g
	K_2HPO_4	1.3 g
	Cobalt chloride	0.3 g
	Agar	15 g
	Ad H_2O_{bidest}	950 ml
	Adjust to pH 6.4 with 10 M KOH	
	Glucose	10 g
	Ad H_2O_{bidest}	50 ml
	Suspend all components except glucose in H_2O_{bidest} and autoclave. Then add filter sterilized glucose solution.	

4.1.11 Software and databases

Databases and local software used in this work listed in Table 4-7

Table 4-7 Overview of web-based and local software used in this work

Software	Description
Delta2D (Decodon, Germany)	2D gel image analysis
DictyBase	http://www.dictybase.org/
Legionella Genome Database	http://genome3.cpmc.columbia.edu/~legion/int_blast.html
NCBI Blast	http://www.ncbi.nlm.nih.gov/BLAST/
Reactome (knowledgebase of biological pathways)	http://www.reactome.org/
KEGG	http://www.genome.ad.jp/kegg/
Vector NTI Advance 10	Invitrogen

4.2 Methods

4.2.1 Growth conditions and storage of bacterial and eukaryotic strains

4.2.1.1 Cultivation and long term storage of bacteria

Bacteria were cultivated either on agar plates or in liquid medium by incubation on shaker as follows:

Legionella spp.: 200 rpm at 37 ºC in YEB+ medium 1-2 days

E. coli: 200 rpm at 37 ºC in LB medium overnight.

Klebsiella aerogenes: 200 rpm at 37 ºC in LB medium overnight.

For long term conservation, *L. pneumophila* and *E. coli* were stored in glycerol stocks, i.e. in broth and glycerol at a ratio of 1:1 (v/v) at -80 ºC. *Legionella* mother plates were generated by plating an aliquot from a glycerol stock on buffered charcoal-yeast extract (BCYE) agar and incubating it at 37 °C with 5 % CO_2 atmosphere for 3 days (Edelstein 1981). These mother plates were kept for 4-6 weeks at 4 °C and used for routinely growing *Legionella* on BCYE agar or in liquid culture for 1-3 days (daughter plates). The number of colony forming units (cfu) was checked by determining the optical density of the culture at wavelength 600 nm (Amersham Pharmacia Biotech Ultrospec 3000) after inoculation to an OD_{600} of 0.1 to 0.3 units

4.2.1.2 Cultivation and long term storage of *D. discoideum*

D. discoideum is an eukaryotic cellular slime mold which is relatively easy to grow. For long term storage *D. discoideum* AX2 strain spores were generated.

To obtain spores, the amoebae were cultivated at 24 °C in 300 ml HL5 medium in Erlenmeyer flasks with shaking at 180 rpm until a cellular density of 3 × 10 cells/ml was reached. Cells were centrifuged for 5 min at 1000 rpm, washed once in 1 x Soerensen buffer, centrifuged again, solved in 5-10 ml 1 x Soerensen buffer and plated on Soerensen agar plates (1.5%). After 48 hours of incubation at 23 °C the mature spores were resuspended in Soerensen buffer and frozen in liquid nitrogen. Due to high rate of spontaneous mutation of *D. discoideum*, a new vial with spores from the stock was thawed every 3 weeks and used to inoculate a 25 ml HL5 medium to obtain preculture.

The *D. discoideum* AX2 strain was routinely grown in HL5 medium. 300 ml of HL5 medium in Erlenmeyer flask was inoculated with 5 ml of preculture and grown with agitation at 23 °C for 3 days. Cell density was determined by taking an aliquot of the culture and counting it in a hemocytometer.

4.2.2 *D. discoideum* plaque assay

For the plaque assay *D. discoideum* cells were collected by centrifugation (1000 rpm, 7 min, at room temperature), washed once with 1 x Soerensen buffer and suspended in infection medium. The final cell density was $1x10^4$ cells/ml. The overnight bacterial cultures of *Legionella* spp. and K. aerogenes were pelleted by centrifugation (3000 g, 5 min, at room temperature) and suspended in 2 ml of sterile H_2O_{bidest}. The bacterial suspension was diluted to 10^9 cells/ml. 100 µl of the *Klebsiella* suspension and 100 µl the *D. discoideum* suspension were mixed with 100 µl of the different *Legionella* strain suspensions respectively and plated onto SM-agar plates. After 3–5 days the plates were examined for plaques formed by *D. discoideum* amoebae.

4.2.3 Screening for *Legionella* mutants defective in arrest of phagolysosomal maturation

This screening allows detecting *Legionella* mutants defective in arresting the maturation of their phagosomes. *D. discoideum* cells of a three day old culture were harvested (1000 rpm, 7 min, at room temperature) and resuspended in the same volume of infection medium (Table 4-8). $25x10^7$ cells were seeded into 25 cm^2 cell culture flasks and the volume was adjusted to 25 ml with freshly mixed infection medium. The final cell density was 1 x 10^6 cells/ml. Before bacterial inoculation the cells were allowed to settle for 30 minutes at 25.5 °C. The *L. pneumophila* Corby transposon mutant library (5700 colonies) was divided into 6 pools. Five day old plate cultures of each pool were suspended in 2 ml of sterile H_2O_{bidest} and the cell density was adjusted to 10^9cells/ml. 250 µl of the prepared bacterial suspensions were added to each cell culture flask at multiple of infection (MOI) 10. Following an invasion period of 3.5 hours, 2.7 ml of colloidal iron particles were added to a final concentration of 1 mg/ml. After 4 hours incubation at 25.5 °C the *D. discoideum* cells were washed three times with 25 ml 1 x Soerensen buffer and once in HB buffer supplemented with a protease inhibitor cocktail. Cells were resuspended in 2 ml of HB and then broken by 12 strokes in a Dura Grind stainless-steel homogenizer. The lysate was subjected to low-speed centrifugation (700 rpm, 5 min at 4 °C) to remove nuclei and unbroken cells. The supernatant was applied to a Miltenyi Biotec MiniMACS column. Then, the column was washed with HB and bound material was eluted with 1 ml of 0.4 % Triton X100 in HB. A 100 µl aliquot of the eluate was immediately plated onto BCYE agar plates supplemented with kanamycin (20 µg/ml). The remaining eluate was frozen at -20 °C.

After 5 days of culturing the bacteria were harvested from the agar plates and selection was repeated. In order to enrich the amount of *Legionella* mutants defective in arrest of phagolysosomal fusion the selection procedure was performed four times.

4.2.4 Haemolysis assay of membrane fractions

For detection of haemolytic activity of membrane fractions, *Legionella* strains were grown on BCYE agar plates. The cells were collected by centrifugation (10 min, 2000 g, 4 °C). The bacterial pellets were resuspended in 20 mM Tris-HCl pH 7.8 (25 °C) and set to an OD of 1.0. Bacteria were then lysed by ultrasonication (3 × 45 s, power cycle 90, power set 65 %, Bandelin25 Sonoplus HD70).

The lysate was subsequently centrifuged (10 min, 2000 g) and pellet resuspended in 1/10 of the original culture volume in 20 mM Tris-HCl pH 7.8 (25°C). 50 µl of all fractions were incubated in stamped out holes of human blood agar plates (30 ml human blood/l in PBS pH 7.4 with 0.9 % agarose) for 24 hours at 37 °C.

4.2.5 Isolation of *Legionella*-containing phagosomes

4.2.5.1 Infection of *D. discoideum* cells

For infection, *D. discoideum* cells, which were grown axenically in HL5 medium for 3 days, were collected by centrifugation for 5 min at 1000 rpm, RT, and suspended in infection medium to final concentration of 1 x 10^6 cells/ml. Three day old *Legionella* culture was suspended in 1 ml sterile H_2O_{bidest} and the cell density adjusted to 5 x 10^9 cells/ml. The experiments were performed at 24 °C in 25 cm^2 cell-culture flasks. 10^8 host cells for each infection were co-incubated with 400 µl *Legionella* culture at a MOI of 20. The colloidal iron particles were added 30 min before harvesting of *D. discoideum* cells to a final concentration of 1 mg/ml.

4.2.5.2 Preparation of iron particles

Colloidal iron particles coated with dextran with an average diameter of 8 nm were prepared as described (Rodriguez-Paris et al. 1993). 10 ml of 1.2 M $FeCl_2$ was mixed with 10 ml of 1.8 M $FeCl_3$ and agitated extensively while 10 ml of 25% NH_3 was added (Table 4-9). Suspension was divided into six aliquots and placed on a magnet until the precipitate had gathered on the bottom of the tube, then the supernatant was decanted. The precipitate was washed once with 5 % NH_3 and twice with deionized H_2O. The sediment was suspended in 80 ml of 0.3 M HCl and stirred for 30 min, after which time 4 g of Dextran (64-76 kDa, Sigma) was added and stirred for a further 30 min. The sample was dialyzed extensively against cold water for 2 days and filtered through the 0.22 µm sterile filter (Millipore, Billerica). The sterile solution was stored at 4 °C for 3 months. The concentration of iron in this solution was approximately 10 mg/ml.

Table 4-9 List of solutions used for iron particle preparation

Solution:	Composition:
1.2 M $FeCl_2$:	4.76 g $FeCl_2 \cdot 4H_2O$ is dissolved in 20 ml of H_2O bidest
1.8 M $FeCl_3$:	9.72 g $FeCl_3 \cdot 6\ H_2O$ is dissolved in 20 ml of H_2O bidest
5 % NH_3:	5 ml 25% NH_3 is added to 20 ml of H_2O bidest

4.2.5.3 Preparation of OptiPrep™ gradient

OptiPrep™ is a ready made, sterile and endotoxin tested solution of Iodixanol, 5,5'-[(2-hydroxy-1-3 propanediyl)-bis(acetylamino)] bis [N,N'bis(2,3dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide], designed for the *in vitro* isolation of biological particles.

For phagosomal isolation the 5-30 % OptiPrep gradient was used. The gradient was prepared by mixing equal volume (4 ml) of 5 and 30 % working OptiPrep solutions in gradient mixer, which consists of communicating cylinders with a diameter of 1 cm. When the gradient was being mixed, the fluid in the "heavy" cylinder was stirred continuously by an inserted screw. The gradient was then poured into polyallomer centrifuge tubes (Beckman) and used immediately. OptiPrep Density Gradient Medium Solutions for 6 separations in 12 ml centrifuge tubes.(table 4-10)

Table 4-10 List of solutions used for OptiPrep gradient centrifugation

Solution:	Composition:
5 % working OptiPrep solution:	22 ml HB buffer mix with 2 ml OptiPrep density Gradient Medium [60% (w/v)]
30 % working OptiPrep solution:	12 ml HB buffer mix with 12 ml OptiPrep density Gradient Medium [60% (w/v)]

4.2.5.4 Purification of bacteria-containing phagosome

Phagosomes were purified according to Lührmann & Haas with modifications (Luhrmann and Haas 2000). All isolation steps were performed on ice. Infected *D. discoideum* cells were harvested and washed twice in 30 ml ice-cold 1x Soerensen buffer and once in ice-cold HB buffer with EDTA-free protease inhibitor cocktail (Roche). Then the cells were suspended in 2 ml of the homogenization buffer containing 5 mg/ml INT, broken by 12 strong strokes in a Dura Grind stainless-steel homogenizer and incubated with Benzonase 50 u/ml for 7 min at 37 ^{o}C. The nuclei and cell debris were removed from the homogenate by centrifugation at 500 × g for 5 min at 4 °C. The lysosomal contamination in obtained postnuclear supernatant was eliminated by running supernatant through the Miltenyi Biotec MiniMACS column placed in the magnetic selector. The

resulting suspension was adjusted to 3 ml by HB buffer, poured on top of 8 ml 5 % to 30% Opti-Prep gradient in homogenization buffer and centrifuged at 100,000 g for 2 hours at 4°C (SW40 rotor, Beckman ultracentrifuge). The bottom of the Polyallomer centrifuge tube (Beckman, California) was pierced by the needle (27 G x ¾''; 0.4 x 20 mm) and gradient was dropped into 0.6-0.8 ml fractions. The phagosomal distribution in Opti prep fractions was assessed by plating serial dilutions on BCYE agar plates to determine the *Legionella*-containing phagosome. The quality of purified phagosomal fraction was controlled by transmission electron microscopy.

Note: The 50 mg of INT (P-iodonitrotetrazolium violet) was solved in 10 ml H_2O to final concentration of 5 mg/ml to give a clear light yellow solution. Gentle heat and sonication were necessary for this procedure. The resulting aqueous solution is reported to be stable for several weeks at 2-8 °C in the dark.

4.2.5.5 Determination of *Legionella*-containing fraction

In order to determine the *Legionella* phagosome containing fraction, a 10 µl aliquot of each fraction was suspended in 90 µl of H_2O_{bidest}, mixed by vortexing, and plated onto a BCYE agar plate. After 3 days of incubation at 37 °C the number of cfu on each plate was determined.

4.2.5.6 Preparation of fractions for transmission electron microscopy

For morphological analysis of the Opti-prep fractions we used conventional glutaraldehyde-osmium fixation. The 25 % glutaraldehyde was added to each sample to a final concentration of 2.5 % for one hour and centrifuged at least 30 minutes or longer until the pellet was formed. The supernatant was removed and the pellet was incubated in 2 % osmiumtetroxid overnight at 4 °C. All samples were then dehydrated with a graded series of ethanol: 50, 70, 80, 90 and twice with 100 %, then twice more with propylenoxid. Subsequently, the pellet was covered by mix of Epon-propylenoxid (1:1) overnight. Following overnight incubation this mixture was replaced by fresh Epon, stained at room temperature for 2-3 hours and then polymerised for a minimum of 3 days at 60 °C.

4.2.6 Preparation of phagosomal proteins

4.2.6.1 Triton X100 treatment

For separation of *D. discoideum* phagosomal membrane proteins from the bacterial fraction, samples were treated with TritonX-100. To the initial volume of each phagosomal fraction, we added 1/20 of 10 % Triton X100 to final concentration of 0.5 %. This mixture was incubated on ice for 30 min and centrifuged for 5 min at 25,000 g, 4 °C to pellet the bacteria.

4.2.6.2 TCA protein precipitation

The proteins were precipitated overnight with 100 % trichloroacetic acid solution (v/v) to a final concentration of 10 %. Proteins were then pelleted by centrifugation at 4 °C, 12 000 rpm for 40-60 min, washed thoroughly (min 8-10 times) with aceton and resuspended in cell lysis buffer. The protein concentration was determined by the Roti-Nanoquant (Roth). The protein solution was stored frozen at −20 C.

4.2.6.3 Estimation of protein concentration using the Roti-Nanoquant (Roth)

The protein concentration was determined by Roti-Nanoquant solution based on modified Bradford's protein assay. The working solution was prepared by dilution of 5 x Roti-Nanoquant solution to 1 x Roti-Nanoquant with $H_2O_{bidest.}$, According to manufacturer's protocols, protein samples in a final volume of 200 µl were mixed with 800 µl of 1 x Roti-Nanoquant working solution and measured at 590 nm and 450 nm against water. A standard graph of 0 to 20 µg BSA was plotted against the quotient A_{590}/A_{450}, to determine protein concentration:

$$\frac{A_{590nm} / A_{450nm} - 0{,}4475}{0{,}1132} = \text{protein concentration [µg]}$$

4.2.6.4 Protein labeling

Phagosomal proteins (5 µg), solubilized in cell lysis buffer, were labeled with Cy3 or Cy5 saturation dyes for scarce samples (Amersham Biosciences) according to the manufacturer's protocol. In brief, cysteine residues in the extracted proteins were reduced by incubating with 2 mM TCEP, at 37 °C for 1 hour (Table 4-11). Then proteins were labeled with 2 mM CyDye DIGE Fluor (Table 4-11) reconstructed in dimethylformamide (DMF), at 37 °C for 30 min. To stop labeling reaction, the double volume of 2 x sample buffer was added. The sample was stored either on ice or frozen at -20 °C in the dark for up to one month.

Table 4-11 List of solutions used for CyDye protein labeling

Solution:	Composition:
2 mM TCEP :	2.8 mg TCEP in 5 ml H2Obidest * TCEP solution is unstable and should be used immediately.
2 mM CyDye DIGE:	100 nmol dye in 50 µl DMF • CyDye DIGE Fluor saturation dye powder must be reconstructed in high quality anhydrous DMF (specification: ≤ 0.005 % H_2O, ≥ 99.8 % pure) open for less than 3 months.

4.2.7 General protein methods

4.2.7.1 SDS Polyacrylamide Gel electrophoresis (SDS-PAGE)

In SDS polyacrylamide gel electrophoresis, proteins are separated as they migrate through a gel on the basis of their molecular weights. SDS is an anionic detergent that denatures proteins. The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions, and substantially unfolds the protein molecules by eliminating the tertiary and secondary structures. Two types of buffer systems are used in protein gel electrophoresis: continuous and discontinuous. In the discontinuous system employed in this work, a non-restrictive large-pore gel called a stacking gel is layered on top of a separating (resolving gel). The buffer composition for the two gel layers differs which in turn differs from the composition of the electrophoresis buffer. At the onset of an electrophoretic separation, the proteins migrate first through the stacking gel and then into the separating gel, where separation takes place. With the aid of a protein marker applied alongside the protein samples of interest, the molecular weight of the proteins applied on the gel can be estimated. The following is the pipetting scheme applied for the preparation of two 12 % acrylamide SDS-gels (Table 4-12).

Table 4-12 List of buffers and solutions used for preparation of acrylamide SDS-gels

Solution:	Stacking gel (5 %), 8 ml	Separating gel (12 %), 15 ml
H_2Obidest	4.5 ml	5 ml
30 % Acrylamide mix	1.3 ml	6 ml
1.5 M Tris-HCl (pH 8,8)	-	3.5 ml
0.5 M Tris-HCl (pH 6,8)	2 ml	-
10 % SDS	80 µl	150 µl
10 % APS	40 µl	75 µl
TEMED	4 µl	5 µl

The electrophoresis system from Bio-Rad was employed in this work and the assembly of glass plates and spacers for the production of the gels was according to manufacturer's instructions. After polymerisation the gel was placed in electrophoresis camera filled up by 1 × Electrophoresis buffer (Table 4-5). For the SDS-gel run, the probes to be analyzed were mixed with the 1 × Laemmli-loading gel buffer, cooked briefly at 95 °C for 5 min and then applied on the gels. Electrophoresis proceeded at an applied voltage of 60 V (or at 20 mA) for 20-40 min before proteins reach the separation gel and then at 90 V for 1-2 hours

4.2.7.2 Urea SDS-PAGE

For resolution of polypeptides with close molecular weight the SDS-PAGE with urea was used as described by Goldsbrough et al. (Goldsbrough et al. 1989). The following is the pipetting scheme applied for the preparation of two 12.5 % acryl amide urea SDS-gels (Table 4-13).

Table 4-13 List of buffers and solutions used for preparation of acrylamide SDS-gels

Solution:	Stacking gel (5 %), 8 ml	Separating gel (12 %)
H_2O_{bidest}	1.495 ml	1.16 ml
30 % Acryl amid mix	570 µl	3.14 ml
1.5 M Tris-HCl (pH 8.8)	-	2.28 ml
0.5 M Tris-HCl (pH 6.8)	825 µl	-
10 M Urea	332 µl	750 µl
10 % SDS	30 µl	76 µl
10 % APS	30 µl	76 µl
TEMED	2 µl	4 µl

4.2.7.3 Protein visualization

Following the SDS-PAGE run, the proteins were visualized by Coomassie dye solution or by silver staining.

Silver staining

Silver staining is the most sensitive non-radioactive method for protein visualisation. It is a complex, multi-step process, which utilizes numerous reagents for which quality is critical. All steps were performed on a shaker with light agitation at room temperature. For mini-gel and large 2D gel near of 50 ml and 250 ml of every solution was used respectively. The gels were incubated for 1-2 hours in fixation solution, washed twice in 50 % ethanol and submerged for 1 min in sensitization solution (Table 4-14). After washing in H_2Odest 2 x 20 sec, gels were placed in silver staining solution for 20 min, washed in H_2Odest 2 x 20 sec and developed for 5-10 min in developing solution. Reaction was stopped by adding 1 % Glycin for 1 min and washing again in H_2Odest for 30 min.

Table 4-14 List of solutions used for silver stained protein visualization

Fixation solution:		
(1-2 hours)	Ethanol 96 %	500 ml
	Acetic acid 100 %	120 ml
	Ad H_2O_{bidest}	1000 ml
	Add 0.5 ml 37 % formaldehyde for 1 l solution before used.	
Sensitization solution:		
(1 min)	$Na_2S_2O_3 \times 5\ H_2O$	0.2 g
	Ad H_2O_{bidest}	1000 g
Silver staining solution:		
(20 min)	$AgNO_3$	2 g
	Ad H_2O_{bidest}	1000 ml
	Add 0.75 ml 37 % formaldehyde for 1 l solution before used.	

Development solution:		
(5-10 min)	Na_2CO_3	30 g
	$Na_2S_2O_3 \times 5\ H_2O$	4 mg
	Ad H_2O_{bidest}	1000 ml
	Add 0.5 ml 37 % formaldehyde for 1 l solution before used.	

Stopping solution:		
(1-10 min)	Glycin	10 g
	Ad H_2O_{bidest}	1000 ml

Coomassie staining

Although Coomassie staining is 50 to 100 fold less sensitive than silver staining, it is a relatively simple and more quantitative staining method. For spots visualisation the gels were fixed in 40 % Ethanol, 10 % acidic acid solution for 1-3 hours, then washed twice in H_2O_{bidest} for 10 min and stained in Colloidal Coomassie solution for 1-7 days (Table 4-15). Then gels were decoloured with H_2O_{bidest} for 1-3 hours, while water was changed several times.

Table 4-15 List of solutions used for Coomassie protein visualization

Coomassie Brilliant Blue stock solution (CBB)	
Coomassie Brilliant Blue G-250	5 g is dissolved in 100 ml of H_2O_{bidest}
Colloidal Coomassie dye stock	
Ammonium sulfate	50 g
Phosphor acid 85 %	6 ml
Ad H_2O_{bidest}	490 ml
CBB	10 ml
Colloidal Coomassie solution	
Colloidal Coomassie dye stock	400 ml
Ethanol	100 ml

4.2.7.4 Pro-Q Diamond Phosphoprotein Gel Staining

Pro-Q Diamond Phosphoprotein Gel Staining is a technology that provides selective staining of phosphoproteins in polyacrylamide gels.

For each 2D gel about 100 ml of every solution was used. All steps were performed with gentle agitation (50 rpm on an orbital shaker) in a container that excluded light. The gels were incubated at room temperature in fix solution for 30 min or overnight, then washed three times in H_2O_{bidest} for 10 min and stained in Pro-Q Diamond Phosphoprotein Gel Stain solution for 60-90 min in the dark. This was followed by incubation in destain solution for 30 min to reduce the gel background signal from non-specific staining and washed 2 x 5 min in H_2O_{bidest} (Table 4-16). Stained gels were visualized by Typhoon 8600 with 532 nm excitation and 580 nm band pass emission filter.

Table 4-16 List of solutions used for Pro-Q Diamond Phosphoprotein visualization

Fixation solution	
Methanol	50 %
Acetic acid	10 %
Destain solution	
1 M Sodium acetate, pH 4.0	50 ml
Acetonitrile	200 ml
H_2O_{bidest}	750 ml

4.2.7.5 Western Blott analysis

Western Blott allows determinion of relative amounts of protein using a specific primary antibody. For immunoblot analysis phagosomal protein samples were loaded with equal concentrations and separated by 12.5 % urea SDS-PAGE. Proteins were electroblotted to nitrocellulose membranes in Towbin buffer (Table 4-5) at 70 mA for 1 hour 15 min (Towbin et al., 1979) and blocked overnight in 1 x PBS with 0.01 % (w/v) Tween-20 and 1 % (m/v) milk. Thereafter the blot was incubated for several hours with the first antibody directed against the protein of interest, followed by washing the blot three times and incubating with the second antibody, conjugated with either alkaline phosphatise (AP) (DakoCytomation Polyclonal Goat Anti-Mouse Immunoglobulins/HRP 1:5000) for minimum 2 hours. Bound antibody was detected by ECL-advance. Stained gels and immunoblots were scanned or detected using a Chemi Lux-Imager (Intas Science Imaging Instruments GmbH, Göttingen, Germany).

Stripping of nitrocellulose membrane

Stripping was used, when more than one protein is investigated on the same blot, or the same protein with different antibodies. For this the membrane was incubated for 30 minutes at 50 °C with slight agitation in stripping buffer, followed by washing three times with PBS/Tween using large volume and re-blocking with milk powder and probes (Table 4-5).

4.2.8 2D gel electrophoresis

Two-dimensional electrophoresis (2D electrophoresis) was first introduced by P.H. O'Farreell and J. Klose in 1975. This method is a powerful tool for the analysis of complex protein mixtures, extracted from cells. This technique sorts proteins according to two independent properties: the first dimension step, Isoelectric focusing (IEF), separates proteins according to their isoelectric point (pI), whereas the second-dimension step, SDS-polyacrylamid gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights. Each spot on the resulting two-dimensional array corresponds to a single protein species in the sample.

The 2D procedure begins with the sample preparation (see 4.2.5). The next step is IPG strips rehydration. IPG strips are provided dry and must be rehydrated with appropriate additives prior to

IEF. First- dimension IEF was performed on a flatbed system at very high voltages with active temperature control. Strip equilibration in SDS-containing buffer prepares the sample for the second-dimension separation. Following equilibration, the strip was placed on the second-dimension gel for SDS-PAGE. The final steps were visualization and analysis of the resultant two-dimensional array of spots.

4.2.8.1 IPG (Immobiline Dry Strip) strip rehydration

24cm Immobiline DryStrip with a nonlinear pH 3–10 gradient using in this study were rehydrated prior to IEF in Immobiline DryStrip Reswelling Tray (Multiphor II).

For analytical gels, a mixture of Cy3- and Cy5- labeled proteins (total 10 µg) and for preparative gels, 500 µg of proteins were solubilized in 450 µl rehydration buffer, containing a few crystals of bromphenol blue (bromphenol blue serves as a tracking dye during IEF and does not interfere with isoelectric focusing.)

The proteins were resolved on a shaker at room temperature. The protective cover was gently removed from IPG strip starting at the acidic end. Strips were then positioned with the gel side down and the pointed end of the strip against the sloped end of the slot, in an effort to prevent trapping of the bubbles under the IPG strip. After that each IPG strip was overlaid with 3 ml DryStrip Cover Fluid to minimize evaporation and urea crystallization. Rehydration was performed at room temperature for 12-18 hours.

4.2.8.2 IEF (Isoelectric focusing)

The first dimension, isoelectric focusing (IEF), was performed using Ettan IPGphor (Amersham Biosciences) at 20 °C. The Ettan IPGphor system is conducted at very high voltage (up to 8000 V) and very low currents (typically less than 50 µA per IPG strip) due to the low ionic strength within IPG strips. During IEF, the current decreases while voltage increases as proteins and other charge components migrate to their equilibrium positions. A typical IEF protocol generally proceeds through a series of voltage steps, which begin at relatively low value.

The strips were put on the surface of an Ettan IPGphor unit with plus end showing away. The pads, wetted by deionized water and blotted with tissue paper, were placed on both ends of IPG strips. Then the electrode strips were carefully put at the ends of the gel covered by wet pads, and strips were covered with Cover Fluid. The following program was used to separate the protein according to their pI: at 500 V for 1 hour, at 1000 V for 1 hour and at 8000 V for 28,000 V·hours. After IEF strips were immediately proceeded to the second-dimension separation or store at -20 °C. The Cover Fluid was recycled through a filter.

Note: The surface of Ettan IPGphor was always cleaned with soft towels without using detergent.

4.2.8.3 IPG strip equilibration

Prior to the second dimension, IPG strips, loaded with saturation labeled samples, were incubated in equilibration solution (table 4-5.) using DTT only, for 10 min at room temperature.

4.2.8.4 Second dimension SDS-PAGE electrophoresis.

Low fluorescence glass plates were used for 2-D DIGE fluorescent gels. All plates were carefully cleaned by ethanol with a soft towel. The gel caster was arranged as described in the manual (Amersham Bioscience). For 6 gels 400 ml acrylamid solution was prepared (Table 4-17).

Table 4-17 List of buffers and solutions used for 2D preparation

12.5 % amid/bisacrylamid total:	**400 ml**
H_2O_{bidest}	66 ml
30 % acrylamid solution	162 ml
2 % bisacrylamid solution	68 ml
1.5 M Tris-HCl (pH 8.8)	100 ml
10 % SDS	4 ml
10 % APS	1 ml
TEMED	200 µl

The acryl amid solution was carefully mixed and poured into the caster up to 1-2 cm below the final desired height. After casting isopropanol was stratified all over the gels in each cassette. The gel was left to polymerise for at least one hour. Meanwhile fresh agarose sealing solution was prepared and cooled slightly. Next, the Immobiline DryStrips were carefully placed between the two glass plates of the gel. By convention the acidic end of the strip is on the left. The strips were gently positioned on the surface of the acrylamid gel, avoiding trapping of air bubbles between the strip and gel. Immediately after applying the Immobiline DryStrip to the second dimension gel, the molten agarose sealing solution was slowly pipetted on the strips preventing bubble formation. The gel cassettes were placed in the Ettan dalt six unit and upper chamber and the top were installed in right direction. Electrophoresis was performed in 1 x SDS electrophoresis buffer at 15 ºC. Gels were run overnight at constant power 6-10 W and stopped before the bromphenol line had left the gel. The analytical gels were scanned immediately after the run, and preparative gels were visualized by Coomassie staining for MALDI-TOF analysis.

4.2.8.5 Gel image analysis

The separated proteins labeled with Cy3 and Cy5 fluorophores were detected on the gels immediately after the second dimension using a Typhoon8600 (Amersham Biosciences). Different data sets were analyzed in order to screen for differences in the amount of the proteins identified on 2D gels. 2D gel image analysis was performed with the Delta2D Software (Decodon, Germany). A set of 18 gels (3 gels for every strain and every time point) was selected for quantitative analysis. Quantitation was based on spot detection and filtering, whole image warping on a reference gel, background subtraction and average gel creation. Only statistically significant changes in spot

representation of 2.0 or higher were analyzed.

4.2.8.6 Protein identification by Mass spectrometry

For protein identification, spots from preparative, Coomassie stained gels were excised from gel using Ettan Spot Picker (Amersham Biosciences). Proteins in the excised gel pieces were digested using in-gel trypsin digest and MALDI-TOF measurements were carried out on a Proteome – Analyzer 4700 (Applied Biosystems, Foster City, CA, USA) as described (Krah et al., 2003). The identification of spots was performed via batch mode using the Mascot protein identification system (Matrix Science, London, UK) applying the recent *D. discoideum* database.

4.2.9 General DNA methods

4.2.9.1 Electrophoresis

The agarose gel was prepared by mixing an appropriate proportion of agarose (to a final concentration of 1 % or 0.8 % for subsequent Southern blot) with 1 x TAE buffer, the mixture cooked and after cooling poured into precast agarose gel chambers. The DNA was then mixed with loading buffer, loaded onto lanes of the gel and electrophoretically separated by voltage application utilizing the 1 x TAE as the running buffer. Following the electrophoretic run, gels were stained in ethidium bromide solution and the DNA visualized under ultraviolet light.

10 x Loading buffer for agarose gels:

Bromphenol blue	0.25 % (w/v)
Glycerol	10 % (v/v)

Ethidium bromide staining solution:

Ethidium bromide	10 g
Ad H_2O_{bidest}	10 ml

4.2.9.2 DNA isolation

Bacterial chromosomal DNA isolation

To isolate the chromosomal DNA, the 2 ml bacterial culture was collected by centrifugation 3 min at 12 000 rpm, washed once in 1 ml TNE buffer and solved in 585 µl TNEX buffer. Then 15 µl of lysozyme (20 mg/ml) was added, and the mixture was incubated for 30 min at 37 °C. After addition of 30 µl of proteinase K (20 mg/ml; Sigma) the mixture was incubated for further 2 hours at 65 °C. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the tube and mixed for 40 min on shaker. The mixture was then centrifuged at 14,000 rpm for 5 min at RT to separate the sample into phases. The upper aqueous layer was removed into a clean tube, carefully avoiding denatured proteins found at the aqueous/chlorophorm interface. The DNA was precipitated from the upper aqueous phase by adding 200 µl 5 M NaCl and 2.5 V of 100 % ethanol and centrifugation

at 13 000 rpm for 10 min. After washing once with 70 % ethanol and once with 96 % ethanol, the pellet was dried for 20-40 min at room temperature and suspended in H_2O_{bidest}.

Plasmid isolation with QIAprep Spin Miniprep kit (Qiagen)

The QIAprep Spin Miniprep kit was routinely used for small scale isolation of plasmid DNA (up to 20 µg). The principle behind it is based on alkaline lysis, coupled with anion-exchange chromatography. The isolation procedure was performed as recommended by the kit's manufacturer.

Determination of DNA concentration and purity

Nucleic acids have a maximum absorption at 260 nm wavelength. The isolated DNA was diluted with distilled water (1:100) and the absorbance at 260 nm against H_2O_{bidest} was measured spectrophotometrically. The calculation of the DNA concentration was based on the following formula:

1 A260 = 50 µg/ml for dsDNA

1 A260 = 33 µg/ml for ssDNA

For determination of DNA purity, the A260/280 coefficient was photometrically determined. An A260/280 < 1.8 indicated contamination of the DNA preparation with protein or aromatic substances; while an A260/280 > 2.0 indicated possible contamination with RNA (LAB FAQs, Roche).

Ethanol precipitation of DNA

Ethanol precipitation of DNA was carried out to remove contaminating salts from a DNA preparation or to concentrate a DNA preparation. The DNA solution was mixed with 1/10 volume of 3 M sodium acetate and 3 volumes of ethanol. The mixture was incubated at -20 °C for 30 min and centrifuged at 14000 rpm for 15 min at 4 °C. The supernatant was removed and the DNA pellet was washed once with 70 % ethanol and twice with 96 % ethanol. The pellet was air-dried and the DNA resuspended in water and stored at -20 °C.

4.2.9.3 Preparation of electrocompetent *Legionella* cells

Electrocompetent *L. pneumophila* Corby cells were prepared by growing cultures on BCYE plates for one day until the bacterial lawn became visible. Bacteria were then washed extensively in ice-cold 10 % glycerol, followed by centrifugation at low speed (4000 g to 6000 g) at 4 °C for 15 min. Next, bacteria were gently resuspended and washed again 2-3 times. Finally, cells were aliquoted in 50 µl volumes and stored on ice for following electroporation or at -80 °C until required.

4.2.9.4 Electroporation

For generation of *L. pneumophila* Corby Transposon library bacterial cells were transformed with

pCDP05a vector by electroporation.

Electroporation with high voltage was achieved with the Gene Pulser II from Bio-Rad. The principle relies on the fact that short electrical impulses directed at bacterial cells generate pores in the cell membrane that facilitates entry of foreign DNA into the cell (Dower et al., 1988).

The settings employed were 25 µF capacitance at 2.3 kV and 100 ohm. After electroporation transformed cells were mixed with 1 ml BYE broth medium and incubated at 37 °C without shaking for 12-16 hours. Bacterial cells were then plated out in 100 - 200 µl aliquots on BCYE-agar plates containing the required antibiotics for selection of recombinants.

4.2.9.5 Enzymatic digestion of DNA with restriction endonucleases

Chromosomal DNA samples for southern blot were routinely subjected to restriction digestions. For a restriction endonuclease reaction, the following components were mixed together and incubated at 37 °C over night. Inactivation of restriction enzyme was carried out by heat treatment at 65 °C for 20 min (Lab FAQs, Roche).

Reaction components	Volumes
Template DNA (20-50 ng)	20 µl
10 x buffer NEB (3)	4 µl
BSA 10 mg/ml	0.4 µl
RNAse A 100 mg/ml	1 µl
Eco RV 20 u/µl	1 µl
H_2O_{bidest}	13.6 µl

4.2.9.6 Polymerase chain reaction (PCR)

The PCR allows the exponential amplification of DNA regions *in vitro* by using a heat-stable DNA polymerase from *Thermophilus aquaticus* (*Tag*). By this method even small amounts of template DNA can be amplified to high copy numbers and easily visualized.

For routine PCR amplification, Tag DNA polymerase kit (QIAGEN) was used. The reaction was performed in a final volume of 20 µl.

The thermal profile was designed according to the annealing temperature of the individual primers and the lenth of the expected amplification product.

a) Protocol

Reaction components	Volumes
Template DNA (20-50 ng)	1 µl
10 x PCR buffer	2 µl
dNTPs (20mM dATP/ dCTP/ dGTP/dTTP)	2 µl
25 mM $MgCl_2$	0.6 µl
Primer 1 (100 pmol/µl)	0.5 µl
Primer 2 (100 pmol/µl)	0.5 µl
Taq polymerase 5 U/ µl	0.5 µl
H_2O_{bidest}	13 µl

b) PCR program

	Number of cycles	Time	Temperature
Denaturation	1	5 min	95 ºC
Denaturation		30 sec	95 ºC
Annealing	25-35	30-60 sec	56 ºC
Elongation		0.5-5 min	72 ºC
Final elongation	1	3 min	72 ºC

4.2.9.7 Southern blot

Digoxigenin-labeling of DNA through PCR

To generate Dioxigenin (DIG) labeled probe for Southern blot hybridization, the following protocol and program for PCR were used:

a) Protocol

Reaction components	Volumes
Template DNA (20-50 ng)	2.5 µl
10 x PCR buffer	5 µl
dNTPs (2 mM dATP/ dCTP/ dGTP and 1.3 mM dTTP)	5 µl
DIG dUTP	3.5 µl
Primer 1 (100 pmol/µl)	1 µl
Primer 2 (100 pmol/µl)	1 µl
Taq polymerase 5 U/ µl	0.5 µl
H_2O_{bidest}	31.5 µl

b) PCR program

	Number of cycles	Time	Temperature
Denaturation	1	5 min	95 ºC
Denaturation		30 sec	95 ºC
Annealing	35	30 sec	56 ºC
Elongation		40 sec	72 ºC
Final elongation	1	8 min	72 ºC

Hybridization

An aliquot of genomic DNA (20-30 µl; ~3-4 µg) was digested overnight with different restriction enzymes. The next day digested DNA was subjected to electrophoresis for 4-6 hours. The gel was then depurinated by incubation for 5 min in depurination buffer, followed by 2 x 15 min incubation in denaturation buffer and 2 x 15 min in neutralization buffer (table 2.5.) The incubations were performed at 25 ^{o}C and the gel was washed in H_2O_{bidest} before changing the buffrs. Finally the gel was equilibrated in 20 x SSC for 10 min. The DNA fragments were then transferred to a nylon

membrane. For this the nylon membrane (Hybond-N^+) was placed on the top of the gel, without trapping air bubbles, then three sheets of 3 mm paper cut to size and wetted with 20 x SSC were placed on top of membrane and covered by 5-7 cm stack absorbent paper towels. The glass plate was placed on the top of the paper towels and weight (approximately 750 g for a 20 cm x 20 cm gel) was applied overnight. Transferred DNA was fixed on the membrane by UV- crosslinking (UV-crosslinker, BioRad). Hybridisation with the probe specific for Km gene was performed at 48 ^oC overnight. Membranes were washed 2 x 5 min at room temperature in low stringency buffer followed by 2 x 15 min at 65 ^oC in high stringency buffer (Table 4-5.).

Detection with Anti-Digoxigenin antibody

After hybridization the membrane blot was briefly rinsed in washing buffer, and then incubated with blocking solution for 30 min at room temperature. This step prevented unspecific binding of the DNA probe to non-homologous DNA regions. The membrane was then incubated for 30-45 min in antibody solution containing the Anti-Digoxigenin antibody conjugated with alkaline phosphatise, washed twice in washing buffer and equilibrated in detection buffer for 2-5 min. Detection was done in a freshly mixed colour substrate solution at 37 °C in the dark (Table 4-18). Once the bands could be visible, the reaction was stopped by the addition of water or TE buffer.

Table 4-18 List of solutions for detection of DNA with Anti-Digoxigenin antibody

Solution	Preparation	Storage/stability
Blocking solution	10 x blocking reagent diluted in Maleic acid buffer 1:10.	Always prepare fresh
Antibody solution	Dilute anti-digoxigenin AP 1:5000 in blocking solution. Centrifuge the antibody for 5 min at 10 000 rpm and pipet necessary amount carefully from the surface.	12 hours at 2-8 °C
Colour substrate solution	Add 200 µl of NBT/BCIP stock solution to 10 ml detection buffer.	Always prepare fresh

4.2.10 DNA array technique

4.2.10.1 DNA-array design

The chip was designed for the detection of pathogenic microorganisms and the effective analysis of its virulence potential. The bacterial pathogens, which are identified by the DNA array are listed in table 8-1. The array was produced based on PCR products of virulence genes, selection markers and antibiotic resistance genes (Table 8-2). The size of each PCR product was approximately 300-500 bp. The primers used to amplify the genes were designed with help of VectorNTI Advance10 with temperature of annealing about 54-55 ºC (Table 8-3, 8-4). Amplification of PCR products

were performed in a 100-µl reaction volume. The concentration and size of each PCR product were verified on agarose gels.

4.2.10.2 DNA-array preparation

For array preparation, nylon membranes (Q filter; Genetix) were wetted in TE solution. Spot blots of PCR products were performed by a Qpix robot (Genetix). Following spot deposition, membranes were fixed for 10 min in 0.5 M NaOH-1.5 M NaCl, washed briefly in distilled water, fixed with UV light and stored dried at room temperature.

4.2.10.3 Labeling of chromosomal DNA with [α-^{33}P]-dCTP

Labeling of 2 µg genomic DNA was performed with 50 µCi of [α-^{33}P]-labeled dCTP (Amersham) and 10 U of Klenow enzyme in 50 µl of reaction volume during 3 hours at 37 ºC.

Reaction components	Volumes
Template DNA (2-3 µg)	10 µl
10 x Klenow buffer	5 µl
dNTPs (5 mM dATP/ dTTP/ dGTP)	1.5 µl
[α-^{33}P]-dCTP, 10 µCi/ µl	5 µl
$pd(N)_{6,}$ 1µg/ µl	0.5 µl
Klenow enzyme, 5U/ µl	2 µl
H_2O_{bidest}	26 µl

Unincorporated nucleotides were removed from DNA-labeling reaction using Microspin ™ G-50 columns (Amersham Pharmacia Biotech). For this, the opened Microspin ™ G-50 columns were centrifuged during 2 min at 4000 rpm and transferred into a fresh centrifugation tube. The radioactive saples were applied onto the columns and centrifuged for further 4 min at the same speed. Radioactivity was measured in both column and flow-through fractions and the percentage of incorporated radioactivity calculated. Labeled DNA substrates were used immediately or stored at 4 °C.

4.2.10.4 Hybridization of DNA array with [α-^{33}P]-dCTP labeled bacterial DNA

High-density arrays were wetted in 2× SSPE and prehybridized for 2 hours in 10-20 ml of a solution containing 5× SSPE, 4% sodium dodecyl sulfate, 1× Denhardt's solution (50× Denhardt's solution is 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin), and 1 mg/ml of denatured salmon sperm DNA (Table 5-2). Hybridization was performed overnight at 65°C. Membranes were washed twice at room temperature and twice at 65 °C in 0.5% SSPE-0.2% sodium dodecyl sulfate. Arrays were then sealed in polypropylene bags and exposed to a phosphorimager screen (Molecular Dynamics) for 24 hours.

5 Results

5.1 Screening for *Legionella* virulence traits by using the *D. discoideum* host model system

5.1.1 Detection of *Legionella* spp. virulence potential by plaque assay

Determination of potential *Legionella* virulence is important for risk assessment of *Legionella* disease.

The plaque assay reveals weather or not the pathogen displays virulence either by evading amoeboid killing or actively killing *D. discoideum.* Predation by *D. discoideum* is scored by plating amoebae on nutrient agar plates seeded with the respective bacterial strains. Successful predation by the amoebae is visualized by the appearance of clear plaques (e.g. food bacteria like *K. aerogenes* or avirulent *Legionella* strains). Absence of plaques reveals

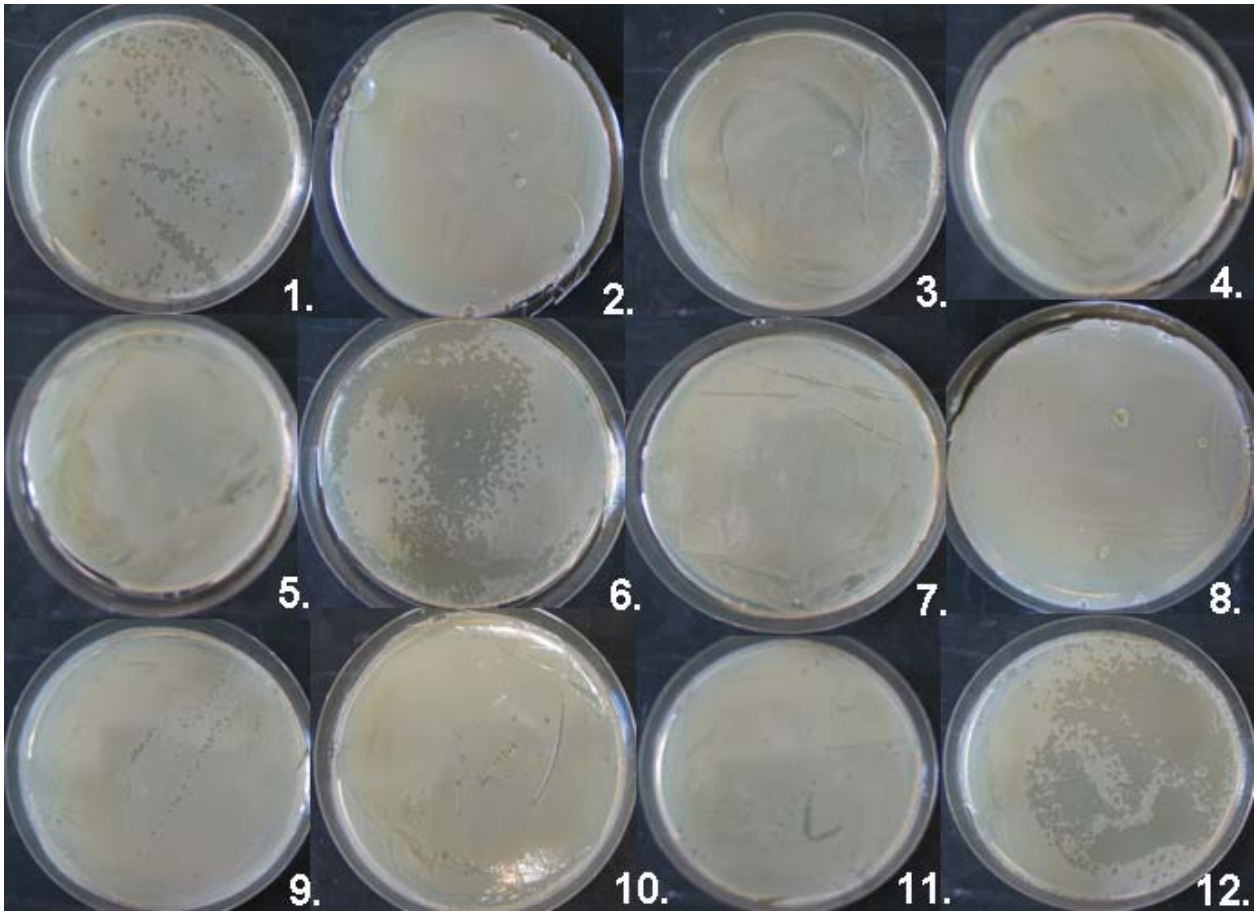


Fig. 5-1 *D. discoideum* plaque assay to screen for bacterial virulence potential
***D. discoideum* cells were plated on SM agar plates seeded either with *K. aerogenes* alone (1); or mixed with *L. micdadei* W02-539 (environmental isolate) (2); *L. micdadei* L01-500 (patient isolate) (3); *L. erythra* (4); *L. bozemanii* (5); *L. hackeliae* (6); *L. longbeacheae* (7);). *L. pneumophila* Corby Sg.1 (8) *L. pneumophila* (patient isolates) (9, 10); LLAP10 (11); *L. lytica* (12)**

resistance to *D. discoideum* predation and may indicate a virulent phenotype.

In order to determine bacterial virulence potential, different *Legionella* spp., including patient and environmental isolates present in Table 5-1 were tested in the plaque assay. *Klebsiella aerogenes* was used in this experiment as a positive control for plaque formation (Fig. 5-1).

On *L. hackeliae* and *L. lytica* bacterial lawn *D.discoideum* cells were able to form numerous plaques. *L. micdadei* W02-539 (environment isolate), *L. erythra*, *L. bozemanii*, *L. pneumophila* (patient isolates) and LLAP10 revealed resistance against amoeba predation and allowed *D. discoideum* to form only few small plaques that may indicate an intermediate virulence phenotype of these bacteria. Three disease-associated strains, *L. micdadei* L01-500 (patient isolate), *L. longbeacheae* and *L. pneumophila* Corby (patient isolates) did not indicate any plaques.

To sum up, the low-pathogenic and non-pathogenic *Legionella* strains revealed no resistance to *D. discoideum* predation, whereas high pathogenic strains and strains associated with human disease did not allow *D. discoideum* to grow and form plaques. Based on this, we can conclude, that the present screen can be used as a test system for evaluation of *Legionella* virulence potential.

5.1.2 Screening for *Legionella* virulence traits by DNA "pathoarray" hybridisation

Microarrays or DNA chip technology is widely used in basic research, drug discovery and diagnostics. It is a useful tool for analysis of gene expression patterns as well as for simmultaneous detecting and typing of bacteria, viruses and other pathogens.

The chip used in this study is hightly suitable for the detection of the presence or absence of genetic sequences, characteristic of specific pathogens, like *Listeria monocytogenes, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium* and others. Bacterial pathogens identified by the present chip are listed in Table 8-1. The array is produced based on PCR products of virulence genes, selective markers and antibiotic resistance genes (Tables 8-2, 8-3, 8-4 supplemented materials). The size of each PCR product was approximately 300-500 bp. The primers used for gene amplification were designed with the VectorNTI Advance10 with annealing temperature of 54-55 ºC (Table 8-3). Amplified PCR products were verified on agarose gels and then spotted onto a nylon membrane.

Besides the other DNA probes, the microarray contains 28 *Legionella* genes, including some components of a putative TISS , TIISS and TIVSSs, transcriptional regulators, and other well-characterised *Legionella* virulence factors (Table 5-1).

Table 5-1 Distribution of *Legionella* specific virulence genes in different *Legionella* strains
Genomic DNAs were isolated from different *Legionella* species: *L. pneumophila* Corby Sg.1 (1); *L. micdadei* W02-539, environmental isolate (2); *L. micdadei* L01-500, patient isolate (3); *L. erythra* (4); *L. bozemanii* (5); *L. hackeliae* (6); *L. longbeacheae* (7); *L. pneumophila*, patient isolates (8, 9, 10); LLAP10 (11); *L. lytica* (12). Isolated DNA was labelled with [33P] dATP and hybridized under high-stringency conditions. (+) define a strong positive signal; (+°) define a weak positive signal.

Gene	Function	1	2	3	4	5	6	7	8	9	10	11	12
flaA	Flagellin	+							+	+	+		
fliA	Sigma factor 28	+			+	+			+	+	+		
flaR	Putative transcriptional regulator	+							+	+	+		
fleQ	Bacterial enchancer binding protein	+							+	+	+		
rpoN	Sigma factor 54	+							+	+	+		
gacA	GacA regulatory protein	+			+	+			+	+	+	+°	
csrA	Global regulator	+							+	+	+		
fleS	Bacterial enchancer binding protein	+							+	+	+		
pilR	Bacterial enchancer binding protein	+							+	+	+		
pilE	Type IV pilin	+							+	+	+		
pilD	Type IV prepilin-like protein specific leader peptidase PilD	+							+	+	+		
fliM	Flagellar basal body gene	+							+	+	+		
flgB	Flagellar basal body gene	+							+	+	+		
motA1	Flagellar motor protein	+							+	+	+		
motA2	Flagellar motor protein	+							+	+	+		
rpoS	Control of stationary phase and stress regulated genes	+							+	+	+		
plaB	Phospholipase A	+			+	+	+		+	+	+	+°	
plcA	Phospholipase C	+						+°	+	+	+		
lipB	Putative lipase	+							+	+	+		
mspA	Zinc metalloprotease	+							+	+	+		
lssD	Putative *hlyD* family secretion protein	+							+	+	+		
lssZ	Putative function	+							+	+	+		
ompR	Outer membrane protein R	+							+	+	+		
dotA	Dot/Icm effector protein	+							+	+	+		
icmR	Required for macrophage killing	+							+	+	+		
fleN	Putative function	+							+	+	+		
flgG	Flagellar basal body gene	+							+	+	+		
traD	Putative type IVA secretion system								+°		+	+	
lvhB10	Putative type IVA secretion system							+	+°		+		
mip	Microphage infectivity potentiator	+		+°			+°		+	+	+	+°	

To determine whether or not these genes are present in *Legionella* spp., genomic DNA was isolated from 12 different *Legionella* species, labelled with [33P] dATP and hybridized under high-stringency or low-stringency conditions (Fig.5-2; Fig.8-1 supplemented materials). The hybridization patterns of four *L. pneumophila* strains and six non- *L. pneumophila* strains among 12 analysed species are presented in Fig. 5-2. The hybridization results are summarized in Table 5-1.

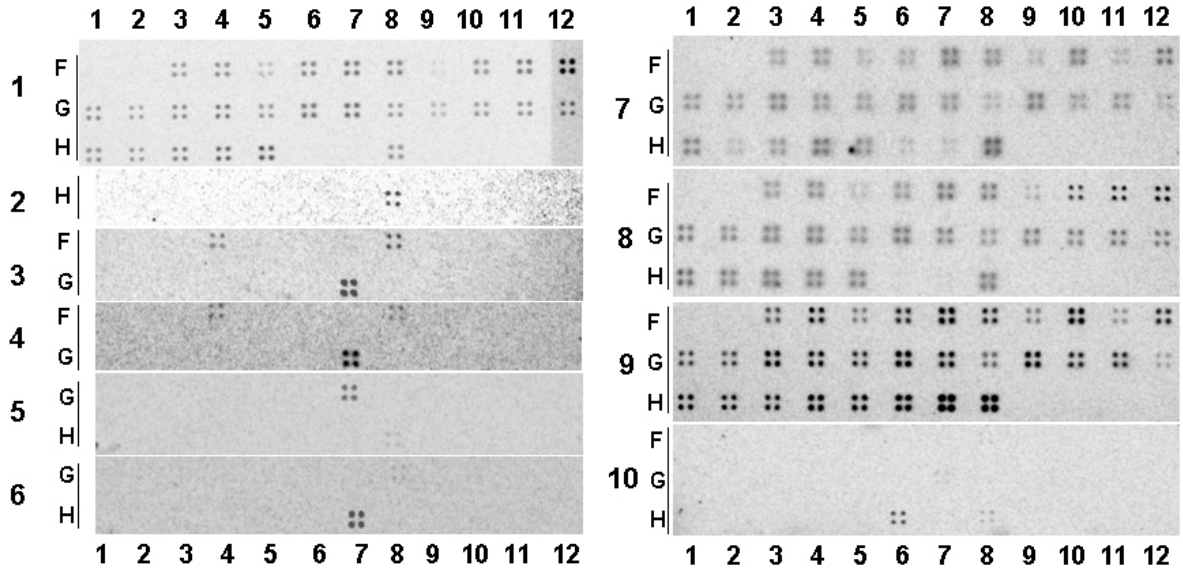


Fig. 5-2 Hybridization of chromosomal DNA from different *Legionella* species with "pathoarray"
The hybridization was performed under low stringency conditions with DNA from *L. pneumophila* Corby Sg.1 (1); *L. micdadei* L01-500, patient isolate (2); *L. erythra* (3); *L. bozemanii* (4); *L. hackeliae* (5); *L. longbeacheae* (6); *L. pneumophila*, patient isolates (7, 8, 9); LLAP10 (10). *L. lytica* and *L. micdadei* W02-539 (environment isolate) did not reveal any hybridisation signal and therefore are not presented. Weak positive signals on the membrane probably indicate the ORFs with low or medium homology to the corresponding genes.

The species which showed no signal after hybridization were not included in Fig. 5-2. Weak positive signals on the membrane probably indicate the ORFs with low or medium homology to the corresponding genes.

In the presented experiment we observed that all *L. pneumophila* strains reveal a full set of virulence genes. The *dot/icm* components were present as predicted only in *L. pneumophila* strains. In contrast the type IVA SS, detected by *traD* and *lvhB10* components, is present not only in *pneumophila* strains, but also in *L. longbeacheae* (*lvhB10*) and LLAP10 (*traD*).

Interestingly, we have identified several virulence-associated factors in non-*pneumophila* strains. First of all, the three non-*pneumophila* strains, *L. micdadei* L01-500 (patient isolate), *L. hackeliae* and LLAP10 display *mip*-like genes. Second, the two strains associated with human pneumonia, *L. erythra* and *L. bozemanii*, possess genes encoding the sigma factor *fliA*, known to mediate contact-dependent cytotoxicity, and the response regulator *gacA*. Finally, *plaB* (encoding phospholipase A) and *plcA* (encoding phospholipase C) genes or ORF with homology to these genes were detected in

8 and 5 of tested strains, respectively (Table 5-1).

Additional hybridization under low stringency condition was performed for screening of ORFs, which have similarity with *Legionella* virulence factors (Fig.8-1 supplementary material). Hybridization patterns of each strain showed a high level of non-specific DNA-DNA interaction. Probably the washing conditions after hybridization are too mild and need to be optimised in future experiments.

Based on available results we can conclude that the present array can be a useful tool for screening of *Legionella* virulence factors, as well as for evaluation of potential pathogenicity.

5.1.3 Determination of haemolytic activity of *Legionella* strains

Haemolytic activity of *Legionella* results in lesion formation in lung tissue during infection. *L. pneumophila* PlaB is a phospholipase with haemolytic activity and has the potential to destroy eukaryotic cells (Flieger et al. 2004). Recently, it was shown that PlaB is expressed before entry into late logarithmic phase and localized within the bacterial membrane fraction (Heuner 2007).

Hybridisation with the „pathoarray" indicated the *plaB* gene in eight of twelve tested *Legionella* strains (Table 5-1). To reveale which of the identified *plaB* genes possess functionally active enzymes, the membrane fractions of analysed *Legionella* strains were checked for haemolytic activity (Fig. 5-3).

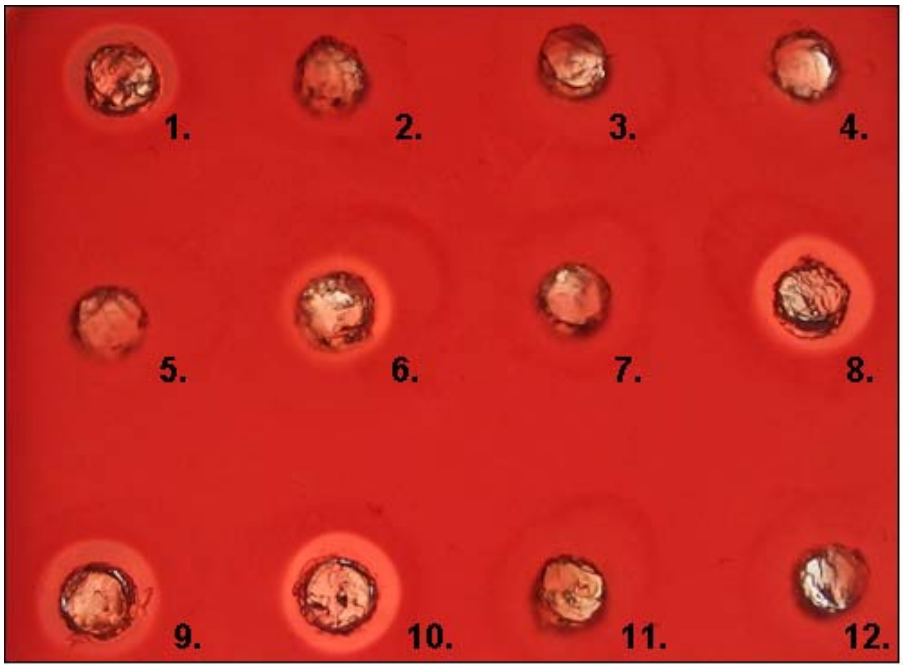


Fig. 5-3 Haemolytic activity of *Legionella* membrane fractions
Different *Legionella* species: *L. pneumophila* Corby Sg.1 (1); *L. micdadei* W02-539 (environmental isolate) (2); *L. micdadei* L01-500, patient isolate (3); *L. erythra* (4); *L. bozemanii* (5); *L. hackeliae* (6); *L. longbeacheae* (7); *L. pneumophila*, patient isolates (8, 9, 10); LLAP10 (11); *L. lytica* (12) were tested. Membrane fractions were isolated and incubated on human blood agar plates for 24 hours at 37 °C. The experiment was performed twice.

Six of eight strains, in which *plaB* gene was detected by hybridization (Table 5-1), caused haemolysis of human red blood cells. The LLAP10, *L. erythra* and *L. bozemanii* strains, which revealed weak *plaB*-specific signal under high-stringency hybridization conditions, had no haemolytic activity in their membrane fractions. This probably indicates that the genes have low homology and, therefore encode a non-functional protein. Nevertheless, it can not be excluded that the haemolytic membrane activity, detected in *L. hackeliae* and *L. pneumophila* patient isolate, depended not only on the phospholipase A activity, but on additional enzyme(s) as well.

To summarize, we have confirmed the functional activity of the phospholipase A, detected by the "pathoarray". Moreover, we can conclude that this activity is present not only in *L. pneumophila* strains associated with the disease, but also in low pathogenic strain like *L. hackelia*e.

5.1.4 Isolation of *Legionella* mutants defective in the arrest of phagosome maturation

Legionella invade and replicate within host cells. They establish protective replicative phagosomes by blocking the acidification of the organelles and interfering with molecular trafficking along the endosomal-lysosomal degradation pathway.

The present mechanical screen for isolation of *Legionella* mutants defective in arrest of their phagosome maturation is based on the assumption that these mutants will be enriched in the late endosomal or lysosomal compartment. The method consists of infection of host cells with a pool of transposon mutants with following host feeding by iron-dextran in order to load the lysosomal compartment. Mechanically lysed cells are then applied onto MiniMACS separation columns in their magnet holder. The flow-throw fraction contains nuclei, mitochondria, ribosomes and other cellular organelles, whereas the eluted fraction, which binds to the column, should be enriched by lysosomes and bacteria enclosed in iron-dextran-containing vacuoles (Fig. 5-4.).

The method was originally applied to the *Mycobacterium tuberculosis*-mice bone marrow macrophages (BMMO) system. Therefore, before starting this screen we evaluated the relevance of the assay for *Legionella- D. discoideum* system. The biogenesis of the *Legionella*-containing phagosome is well described in macrophages. However, it is still unknown at what time after infection *Legionella* precede their replication compartment by avoiding lysosomal degradation in *D. discoideum* cells. Therefore, to optimise the mechanical screening we have calculated the percentage of bacteria associated with the lysosomal fraction during *D. discoideum* infection.

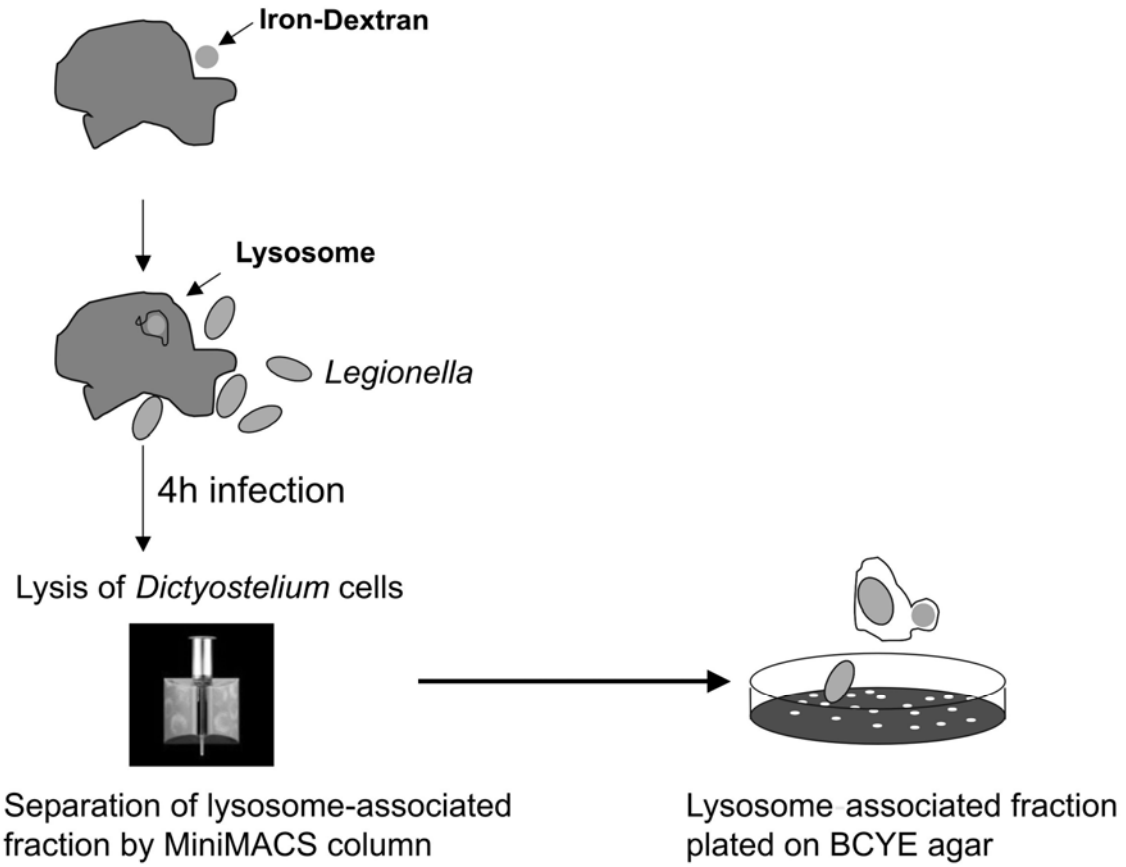


Fig. 5-4 Isolation of *Legionella* mutants defective in the arrest of phagosome maturation
***D. discoideum* cells are incubated with iron-dextran that chases into lysosomes. After infection with *Legionella* from a library of transposon-mutagenized bacteria and host cell lysis iron-dextran-loaded lysosomes are separated by MiniMACS columns. The lysosome-associated bacterial fraction is cultivated on BCYE agar. After four rounds of enrichment, the majority of mutants should reveal the desired phenotype.**

For this, we have chosen two *Legionella* species *L. pneumophila* Corby and *L. hackeliae,* which reveal different phenotypes during infection of *D. discoideum*. *L. pneumophila* Corby has a high replication rate and does not inhibit acidification of the phagosomal compartment (intraphagosomal pH 6.33). In contrast, *L. hackeliae* does not reveal a pathogenic phenotype and allows the phagosomal compartment to acidify (intraphagosomal pH 4.06)(Haegele 2002).

The *D. discoideum* cells loaded with iron dextran were infected with described strains. After 2, 4, 12 and 24 hours post infection cells were lysed and applied onto MiniMACS separation column. The percentage of bacteria associated with the lysosomal fraction was calculated by the ratio of bacteria in the column-bound fraction to the total amount of bacterial cells (Fig. 5-5).

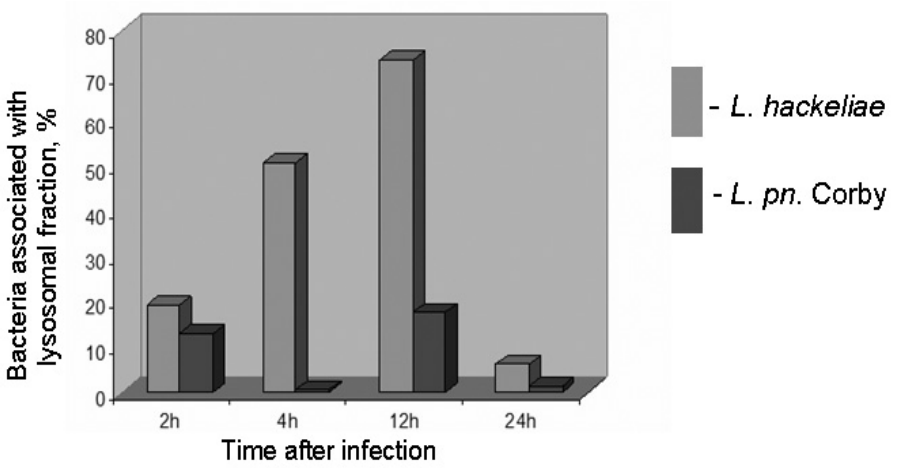


Fig. 5-5 Percentage of *Legionella* associated with lysosomal fractions during infection
Infected *D. discoideum* were lysed after 2, 4, 12 and 24 hours post infection and subjected to separation on MiniMACS™ separation columns. Eluted fractions, containing lysosome and lysosome-associated organelles were plated onto BCYE agar plates and the numbers of bacteria colony forming units were calculated. The total amount of bacteria was calculated by plating an aliquot of lysed *D. discoideum* onto BCYE agar plates. The experiment was performed twice.

The population of phagolysosomal *L. hackeliae* increases from 20 % to 76 %. However, at 24 hours post infection we observed a bacterial decrease in the lysosomal compartment (7 %) which probably indicates that *L. hackeliae* was particulary digested. At 2 hours post infection no more than 12-16 % of *L. pneumophila* Corby was found to be lysosome-associated and at 4 hours the number of lysosome-associated pathogenic bacteria was 100 fold less compared to the non-pathogenic one. This experiment suggested that it is possible to separate *Legionella* with different phenotypes, and that 4 hours after infection is the best time point for performing the screening in *D. discoideum* cells.

Thus, we concluded that the mechanical screen is useful for isolation of *Legionella* mutants defective in phagosome maturation arrest.

5.1.4.1 Generation of a *Legionella* transposon library

To obtain *L. pneumophila* Corby random mutants, the mini-Tn10 mutagenesis was applied (Pope et al. 1994). The delivery vector, pCDP05a, contains a mini-Tn10 element which encodes a kanamycin resistance (Km) gene and altered target specificity (ATS) transposase which markedly increase the randomness of transposition.

L. pneumophila Corby was electroporated with the transposon mutagenesis vector pCDP05a, and the transposition mutants were selected.

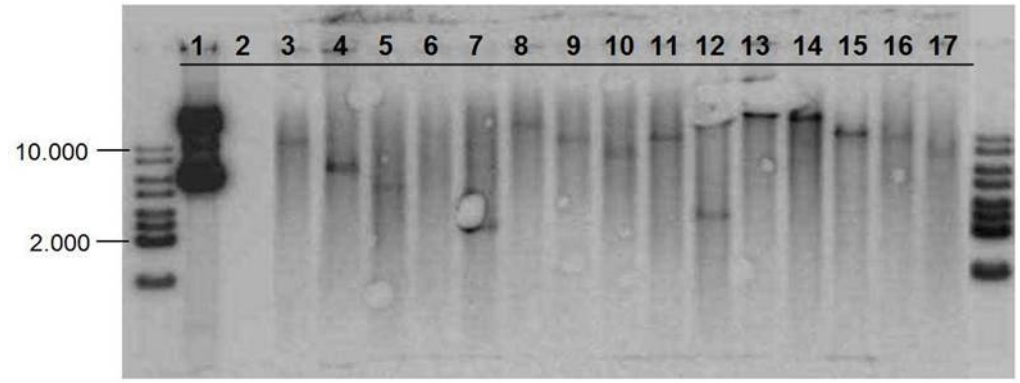


Fig. 5-6 Southern blot analysis of representative *L. pneumophila* Corby::Tn10 clones
Genomic DNA was digested with EcoRV and probed for hybridization with a Km-probe. Fifteen mutants were picked randomly (lines 3–17). *L. pneumophila* Corby DNA (WT) was included as a control and as expected showed no hybridization signal (line 2). Molecular masses are indicated in kilobases.

Since the vector contains the transposon with a Km^R gene for positive selection and a *sacB* gene for counterselection, the Km^R and Sac^R bacteria potentially had a chromosomal insertion with loss of the delivery vector. To test this, we examined the presence of the Km^R gene by Southern blot hybridization of 15 randomly picked Km^R and Sac^R colonies (Fig.5-6). Importantly, each strain possessed a uniquely-sized hybridizing band that confirmed random insertion of miniTn10 in *Legionella* chromosome.

Individual mutant colonies (5600) were picked from plates, cultured and stored in glycerol in 96-well plates. The library was divided into six pools of 960 mutants. Bacteria in each pool were mixed together and subjected for screening. After 4 rounds of selection, 48 randomly picked colonies were cultivated and processed for Southern blot hybridisation to identify potential mutants' families with the same genotype.

Obtained mutants are potentially involved in manipulation of host signalling pathways by which *Legionella* avoid phagolysosomal degradation. We plan to identify the localisation of the transposon insertion and characterize these mutants.

5.2 Phagosomal proteome of *Legionella*-infected *D. discoideum*

5.2.1 Isolation of *Legionella*-containing phagosomes

The *Legionella* specific phagosomes avoid fusion with the lysosome, allowing intracellular replication of bacteria. The components of a bacterial type IV secretion system were shown to play a critical role in this process. However, the host factors, which regulate maturation of the phagosome, are largely unknown. Therefore, the detailed characterization of *Legionella*-containing phagosome composition is important for better understanding of molecular mechanisms taking place during infection.

To obtain the phagosomal enriched fraction a protocol for isolation of this *D. discoideum* organelle, infected by *Legionella,* was established. The method was based on a previously described protocol and was optimized for high purity (Luhrmann and Haas 2000). The newly established protocol was used for analysis of phagosomal protein changes during *Legionella*-infection.

The infection of axenically grown *D. discoideum* AX2 cells was performed as previously described (Ünal and Steinert, 2006). The isolation of *Legionella* containing phagosomes was performed at 2, 4 and 6 hours post infection since most fateful cellular changes occur during this time frame. The method starts with infection of *D. discoideum* cells by *Legionella*, and subsequent removal of extracellular bacteria by several washing steps. Infected *D. discoideum* cells were then lysed

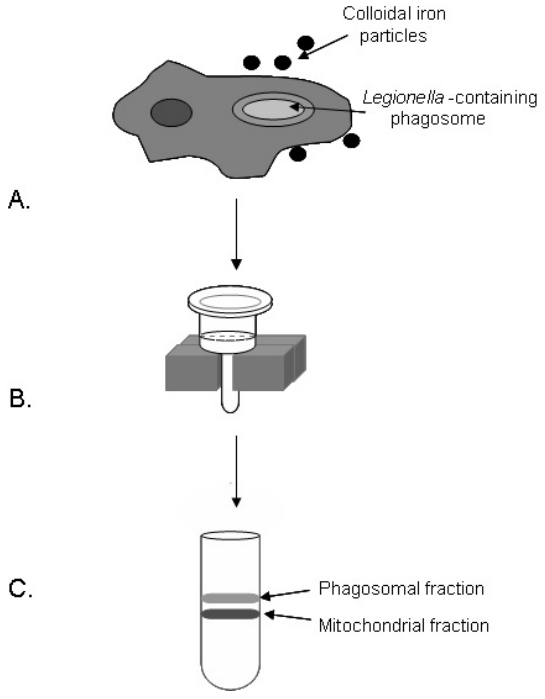


Fig. 5-7. Schematic illustration of phagosome isolation procedure. The isolation method starts with infection of *D. discoideum* by *Legionella* and consist of following steps:

A. Loading the lysosomal compartment of infected cells by colloidal iron particles, removal of the nuclear and nucleic acids from cell lysate and treatment by INT in order to increase the density of mitochondrial fraction.

B. Lysome elimination on Miltenyi Biotec MiniMACS column placed in the magnetic selector.

C. Fractionation by Opti-prep gradient for separation the mitochondria and phagosomal organelles.

mechanically by strokes in a Dura Grind stainless-steel homogenizer. Unbroken cells and whole nuclei were removed by centrifugation and the obtained postnuclear supernatant was treated with Benzonase, an enzyme mixture for nucleic acid degradation. This step leads to a more liquid consistency of the sample and permitting separation of phagosomes from other organelles. Lysosomes were eliminated on MiniMACS separation column by colloidal iron loading prior the isolation procedure (Fig. 5-7).

Mitochondrial contamination was eliminated by iodophenylnitrophenyltetrazolium (INT) "heavy labelling". Succinate dehydrogenase activity of mitochondria reduces the INT dye and the product (formazan) is deposited on mitochondrial membranes. This step increases the density of these organelles after treatment and allowing separation of bacterial phagosomes from mitochondria by density gradient centrifugation in a discontinuous Opti-prep gradient (Fig. 5-9C).

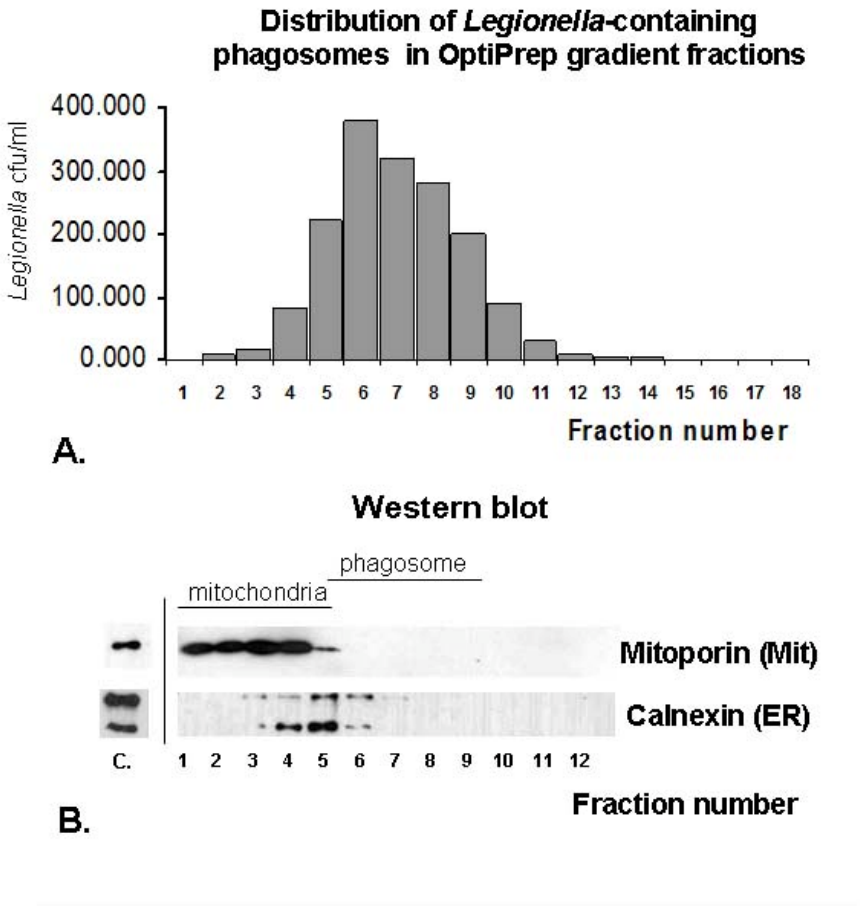


Fig. 5-8 Distribution of *Legionella*-containing phagosomes in OptiPrep gradient fractions
A. Each fraction was plated onto BCYE-agar plates and CFU of *Legionella* were counted. The highest bacterial numbers were found in the phagosomal fractions.

B. Immunoblot analysis of OptiPrep fractions with well-characterized organelle markers. Mitochondria were detected by monoclonal α-mitoporin-antibody; ER was detected by calnexin-specific antibody. C.-control represents the whole D. *discoideum* lysate. 1-12- number of OptiPrep gradient fractions.

The distribution of *Legionella*-containing phagosomes in the gradient was analyzed by plating 18 separate Opti-prep fractions onto BCYE agar plates and counting bacterial CFU/ml (Fig.5-8A). A number of additional analyses were performed to assess the purity of the phagosome preparation. The separation of organelles by ultracentrifugation was confirmed by immunoblot analyses (Fig. 5-8B). For immunoblot analyses the ER marker calnexin, which was shown to be present in *Legionella*-containing vacuoles, and the mitochondrial marker mitoporin were used. Additional Western blot analysis with the VatA (subunit of vacuolar ATPase) antibodies did not reveal the presence of this protein in sucrose gradient fractions (data not shown). In all immunoblot experiments whole *D. discoideum* proteins lysate was used as a positive control.

The morphological analysis of the respective OptiPrep fractions by standard transmission electron microscopy procedures showed that "early" ("heavy") fractions contain mitochondria and different types of vesicles. Fractions corresponding to the highest counts of *Legionella* usually reveal single bacteria located in "spacious" vacuoles (Fig. 5-9A-C). In addition, electron microscopy of the magnetically removed lysosomal fraction confirmed successful elimination of these organelles from the phagosomal preparation (Fig.5-9D).

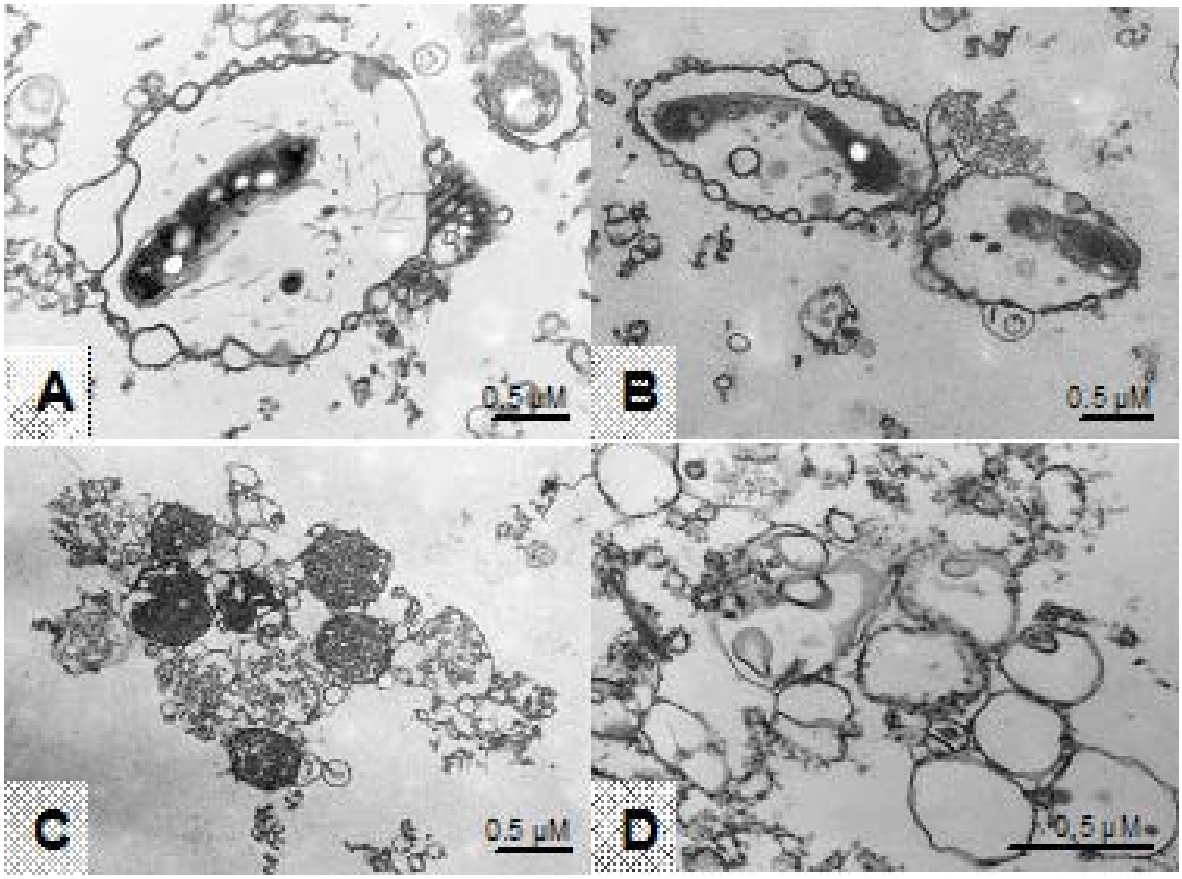


Fig. 5-9 Transmission electron microscopy of OptiPrep fractions collected after ultracentrifugation
***D. discoideum* cells were infected with *L. pneumophila* Corby for 2 hours. After separation by ultracentrifugation different organelle fractions were analysed.**
A. and B. *Legionella*-containing phagosomes are surrounded by host vesicles
C. Mitochondrial fraction shows the separation of this organelle from phagosomal fraction.
D. Lysosomal fraction eliminated by magnet.

Degradation processes in the phagosomal preparations were excluded by one-dimensional SDS-PAGE analysis (data not shown).

Here we present a method for purification of the bacteria-containing phagosome with minimal endocytic organelle contamination. This method is useful for further analysis of this organelle by different molecular biological methods, including the determination of the protein composition by 2D gel electrophoresis.

5.2.2 Analysis of the phagosomal proteome

5.2.2.1 Identification of the phagosomal protein composition

The host phagosomal proteins are predicted to be the targets for *Legionella* secreted effectors. Therefore, their identification is of special interest.

In order to obtain the map of phagosomal proteome, we exclude the bacteria from host phagosomal membrane by detergent treatment with subsequent sedimentation of the bacteria. With the migration conditions used in the present study, the phagosomal proteins loaded on 2-D gels yielded patterns displaying spots between 13 and 95 kDa in size with pI (isoelectric point) values ranging from 4.6 to 10.1. The preparative Coomassie-stained gel (two replicas) of the phagosomal proteins isolated from *D. discoideum* after 2 hours of infection with *L. pneumophila* Corby was used to display the identified proteins and build up our database (Fig. 5-10). This data base was used for temporal- and species-specific analysis of the phagosomal protein composition (table 5-2).

A total of 440 visible spots were systematically excised for MALDI (Matrix Assisted Laser Desorption/Ionization) –TOF (Time-Of-Flight) MS (Mass Spectrometry) and mapped by MASCOT to 157 proteins on the basis of the *D. discoideum* genome database (http://dictybase.org/). MALDI-TOF MS method is based on the principle that a temporally and spacially well defined group of ions of different mass/charge (m/z) ratios, is subjected to the same applied electric field and allowed to drift in a region of constant electric field. They traverse this region in a time period that depends upon their m/z ratios.

With help of MALDI-TOF MS the 142 spots were identified as proteins or open reading frames with a known or a predicted function. The putative function of the remaining 15 “hypothetical proteins” is unknown. Table 5-2 provides a list of all proteins including their accession number, known or predicted function, molecular weight and pI.

The identified phagosomal proteins are from different subcellular compartments and belong to a variety of functional groups (Fig. 5-11). The largest group comprises 36 proteins which are involved in protein biosynthesis and catabolism. Seven tRNA synthetases, six elongation factors and several ribosomal proteins are shown to be associated with the phagosome enriched fraction. The 31 proteins of the second largest group are members of the metabolism family. We have

detected eight proteins participating in purine and pyrimidine biosynthesis, and three enzymes that synthesize fatty acids. Seven other proteins, including phosphoenolpyruvate carboxykinase, glucose 6-phosphate-1-dehydrogenase, aldehyde dehydrogenase are involved in carbohydrate metabolism.

The cytoskeleton and signal transduction class comprises 28 proteins. Actin, cofilin, the actin-like protein ArpH and coronin, were all shown to be involved in actin nucleation. Several other detected proteins, such as coastosin, severin, actin caping and binding proteins, are directly or indirectly implicated in actin rearrangement and phagocytosis. An additional candidate for organization of the actin cytoskeleton was the actin-binding protein hisactophilin, which can be found in two isoforms in *D. discoideum* phagosomal fractions. Seven of the detected proteins are involved in signal transduction. These include *rdiA, pkiA*, *fttB* (coding the proteins with protein kinase inhibitor activity), *cdcD* (cell division cycle protein 48) and *pgkA* (phosphoglycerate kinase) with calmodulin binding and regulating function. The 13 stress response proteins were sorted to individual classes. These include *aif*, encoding the apoptosis inducing factor, protein phosphatase 2C homolog 1, responsible for osmotic stress, the A and B subunits of ribonucleoprotein vault, the stress-induced sti1-like protein and other proteins mentioned in the table (Table 5-2)

Since we expected the presence of some *Legionella* proteins in the phagosomal preparation, the obtained mass spectrum results were screened for respective peptides in the *L. pneumophila* genome data base (http://*Legionella*.cu-genome.org). Four proteins (FAD linked oxidase, TolB colicin import protein, adenosylhomocysteinase and (exo) ribonuclease R) had a high score and therefore a high probability to be present in the phagosome. Nevertheless, the calculated pIs of FAD linked oxidase, TolB colicin import protein and (exo) ribonuclease did not coincide with pIs of correspondent protein spots in the gel. Therefore, we assume that these are *D. discoideum* proteins. However, there is one protein, which has been identified as S-adenosyl-L-homocysteine hydrolase, that can not formally be excluded as a *Legionella* protein.

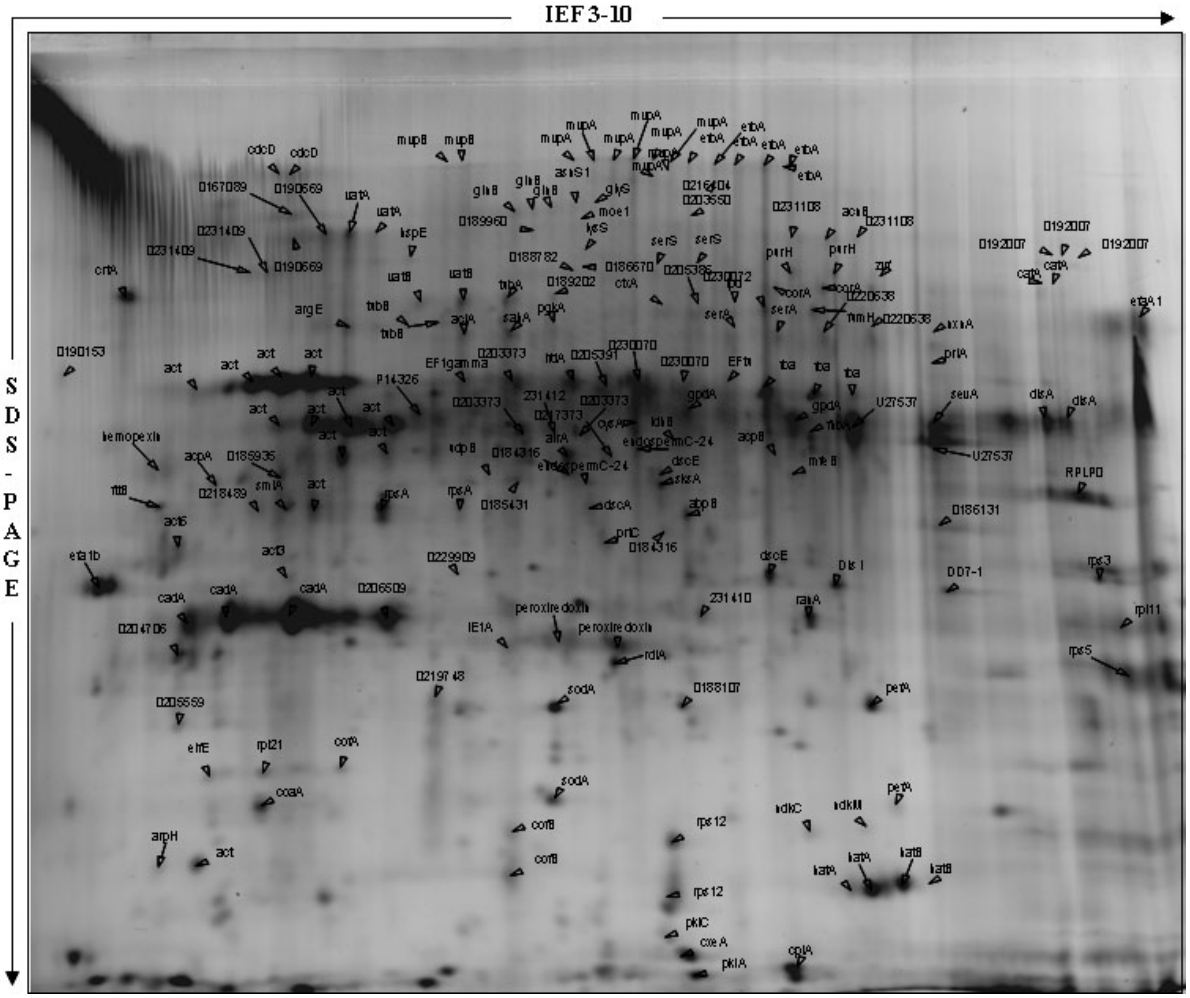


Fig. 5-10 High-resolution 2D gel of purified phagosomal proteins isolated from *D. discoideum* after 2 hours of infection with *L. pneumophila* Corby
Proteins were separated according to their isoelectric point and then by standard SDS-PAGE. After Coomassie staining the major protein spots were analyzed by MALDI-TOF MS.

Table 5-2 List of phagosomal proteins identified by MALDI MS

Protein Name	Acc N.	Description	MW	PI
Metabolism (31)				
Carbohydrate metabolism (8)				
ALDH	DDB0231504	aldehyde dehydrogenase	55,16	5,78
AlrA*	DDB0215363	aldehyde reductase	33,62	6,31
BC4V2_0_01631	DDB0186848	putative glucosamine-6-phosphate isomerase	82,64	6,14
DDB0231108	DDB0231108	Phosphoenolpyruvate carboxykinase	62,53	6,51
Fba	DDB0231387	fructose-bisphosphate aldolase	38,88	7,01
GpdA	DDB0185087	glyceraldehyde-3-phosphate dehydrogenase	36,53	6,46
IdhC	DDB0231401	isocitrate dehydrogenase (NADP+)	46,34	6,36
Zwf	DDB0231285	glucose 6-phosphate-1-dehydrogenase	56,66	7,16
Fatty acid metabolism (3)				
DDB0166993	DDB0166993	propionyl-CoA carboxylase beta chain	60,65	6,82
FcsA	DDB0191105	fatty acyl-CoA synthetase	74,56	6,03
PccA	DDB0230063	propionyl-CoA carboxylase	79,77	6,29
Vitamins Co-enzymes & Hormones (1)				
JC3V2_0_00291	DDB0205386	ATP citrate lyase β-subunit	50,23	6,05
Purines & Pyrimidines (8)				
BC4V2_0_00885*	DDB0186131	similar to ribose-phosphate pyrophosphokinase	37,10	7,68
DDB0230174	DDB0230174	adenosine kinase	56,27	5,57
DDB0230202	DDB0230202	5'-nucleotidase	34,44	6,09
DDB0231470	DDB0231470	urate oxidase	33,01	8,95
GuaB	DDB0230098	IMP dehydrogenase	44,72	7,97
Pyr56	DDB0214958	pyrimidine base biosynthesis	35,37	6,21
ThyA	DDB0214905	thymidylate synthase (FAD)	32,36	6,44
UdpB*	DDB0230170	uridine phosphorylase	33,01	5,69
Amino acid biosynthesis & catabolism (11)				
AsnA	DDB0230140	asparagine synthetase	63,45	5,9
DDB0230064	DDB0230064	sulfate adenylyltransferase	65,58	6,18
DDB0230070	DDB0230070	S-adenosylmethionine synthetase	41,74	6,14
DDB0230072	DDB0230072	Serine hydroxymethyltransferase	50,66	6,54
DDB0231438	DDB0231438	glutamate dehydrogenase [NAD(P)+]	55,01	7,98
glnB*	DDB0231551	glutamate-ammonia ligase	82,56	5,8
JC2V2_0_03178	DDB0203373	4-hydroxyphenylpyruvate dioxygenase	41,97	5,77
Lpd	DDB0216232	Dihydrolipoamide:NAD oxidoreductase	51,76	6,88
Pyd3	DDB0185221	beta-alanine synthase	44,06	6,32
SahA	DDB0191108	S-adenosyl-L-homocysteine hydrolase	47,25	5,81
SerA	DDB0230052	3-phosphoglycerate dehydrogenase	44,72	6,61
Protein biosynthesis & catabolism (37)				
Protein biosyntesis (15)				
BC4V2_0_01413	DDB0186657	regulation of translational initiation	37,01	6,56
BC5V2_0_01173	DDB0219464	elongation factor Tu	46,60	7,2
DDB0192007	DDB0192007	poly(A)-mRNA binding protein	62,74	8,83
Efa1B	DDB0191174	elongation factor 1 beta	24,11	4,54
EfaAI*	DDB0191135	elongation factor 1 alpha	49,63	9,07
EifE	DDB0191442	eifE	17,08	4,95
EfbA*	DDB0191363	elongation factor 2	92,60	6,22
IfdA	DDB0191262	Similar to ATP-dependent, RNA helicase	44,32	6,04

Moe1*	DDB0219927	eIF-3 zeta	60,26	5,67
Rpl11*	DDB0214853	ribosomal protein L11	22,38	10,12
RPLP0	DDB0219977	ribosomal acidic phosphoprotein P0	32,83	9,01
Rps12*	DDB0230044	Ribosomal Protein Small subunit	14,90	6,73
Rps3	DDB0201667	ribosomal subunit	24,20	9,83
Rps5	DDB0230022	Ribosomal Protein Small subunit	21,20	9,93
RpsA*	DDB0230016	40S ribosomal protein SA	27,10	5,29
tRNA synthetase (7)				
ArgS1	DDB0231324	arginyl-tRNA synthetase	67,00	6,43
AsnS1	DDB0231308	asparaginyl-tRNA synthetase	64,89	6,1
CysS	DDB0231318	cysteinyl-tRNA synthetase	74,62	6,63
GlyS	DDB0231313	glycyl-tRNA synthetase	76,17	6,44
LysS	DDB0231263	lysyl-tRNA synthetase	61,96	5,8
SerS	DDB0231305	seryl-tRNA synthetase	51,55	6,13
ThrS1	DDB0231248	threonyl-tRNA synthetase	81,14	6,66
Protease & peptidase (9)				
ArgE	DDB0191165	acetylornithine deacetylase	49,04	5,28
BC5V2_0_01884*	DDB0189202	Metalloproteinase	47,72	6,3
JC1V2_0_01800	DDB0190923	similar to methionine aminopeptidase 2	46,58	6,52
JC2V2_0_02854	DDB0168337	26S proteasome, triple-A ATPase subunit1	47,37	6,1
PrlA	DDB0204785	proliferation associated protein	38,14	6,47
PsmC1	DDB0202018	TAT-binding, 26S protease subunit homolog	49,16	5,9
PsmC3	DDB0186002	26S proteasome ATPase 3 subunit	46,84	5,36
TbpB	DDB0191435	HIV1 TAT-binding protein	45,51	6,45
CpiA *	DDB0220657	cystein proteinase inhibitor	10,41	6,4
Protein folding (6)				
JC2V2_0_03169	DDB0169209	chaperonin containing TCP-1 zeta subunit	59,35	6,2
DisA	DDB0185040	protein disulfide isomerase	39,88	8,52
DscE*	DDB0215382	discoidin II	28,56	6,65
JC1V2_0_01800	DDB0190923	similar to methionine aminopeptidase 2	46,58	6,52
JC2V2_0_01412	DDB0217373	similar to discoidin I, A chain.	28,24	6,2
Tcp	DDB0191128	t-complex polypeptide 1 homologue	59,37	6,55
Oxidoreductase activity (5)				
BC5V2_0_01017	DDB0188339	Putative amino oxidase	52,33	6,07
BEC6V2_0_00612*	DDB0219748	oxidoreductase activity	94,79	6,06
JC2V2_0_01686	DDB0217516	peroxiredoxin 4	22,95	6,09
MfeB	DDB0214811	hypothetical peroxisomal multifunctional enzyme	32,38	6,4
SodA*	DDB0191290	superoxide dismutase	15,85	5,85
Vacuolar H + ATPase (5)				
BC4V2_0_00187	DDB0185431	ATP synthesis coupled proton transport	33,11	6,86
BEC6V2_0_00550	DDB0184316	ATP synthase F1 subunit alpha.	33,61	7,71
JC1V2_0_01541	DDB0190669	ATP synthase beta-subunit	70,80	5,62
VatA*	DDB0201563	vacuolar H+-ATPase A subunit	68,16	5,35
VatB*	DDB0185207	vacuolar H+ ATPase B subunit	54,84	5,53
Mitochondrial proteins (10)				
BC4V2_0_00930	DDB0218638	lysine-ketoglutarate reductase activity	102,91	6,19
BC5V2_0_00456	DDB0187790	succinyl-CoA:3-ketoacid-coenzyme A transferase	54,90	8,07
CxeA*	DDB0191104	cytochrome c oxidase subunit V	13,5	6,6
DDB0220638	DDB0220638	citrate synthase	51,18	8,09

FumH*	DDB0231400	fumarate hydratase	50,69	6,81
GpdA	DDB0185087	glyceraldehyde-3-phosphate dehydrogenase	36,53	6,46
IdhB	DDB0231294	isocitrate dehydrogenase	38,66	8,48
MdhB	DDB0230188	malate dehydrogenase	37,62	8,78
NdkC	DDB0185051	nucleoside diphosphate kinase		
NdkM*	DDB0214817	nucleoside diphosphate kinase	23,98	9,69
SdhA	DDB0214886	succinate dehydrogenase	68,47	6,46
Cytoskeleton & signal transduction (28)				
AbpB	DDB0214810	actin bundling protein	33,33	6,19
AclA *	DDB0219936	component of the Arp2/3 complex	46,67	5,72
AcpA*	DDB0191202	actin capping protein	30,68	5,02
AcpB*	DDB0191243	actin capping protein	31,14	6,84
Act*		Actin	41-43	5.1
ArpH	DDB0191138	component of the Arp2/Arp3 complex	15,13	5,05
CadA	DDB0191175	calcium-dependent cell adhesion molecule-1	23,91	5,4
CdcD*	DDB0191154	cell division cycle protein 48	88,50	5,19
CoaA*	DDB0215369	Coactosin	15,99	5,25
CofA*	DDB0214987	Cofilin	15,21	6,29
CorA*	DDB0191115	Coronin	49,18	6,81
CrtA	DDB0191384	calreticulin, Ca^{2+}-binding protein	48,33	4,63
CtxA*	DDB0191103	Cortexillin I	50,50	6,2
CtxB	DDB0185031	Cortexillin II	50,43	5,5
FttB*	DDB0190707	Protein kinase C inhibitor activity	27,74	4,78
GpbA	DDB0185046	G protein b-subunit	38,60	6,31
HatA	DDB0215335	Hisactophilin	13,45	6,89
HatB	DDB0215336	hisactophilin II	13,63	7,08
JC2V2_0_03244	DDB0203397	actin bundling protein	56,71	5,56
NxnA*	DDB0191502	annexin VII	46,48	7,66
GkA	DDB0191349	Phosphoglycerate kinase, calmodulin-binding	45,68	6,01
PkiA*	DDB0216234	similar to protein kinase C inhibitor	14,05	6,58
RanA	DDB0215409	GTP-binding nuclear protein Ran	24,03	6,96
RdiA*	DDB0216235	Rho GDP-dissociation inhibitor	22,33	6,0
SevA	DDB0188380	Ca^{2+} -dependent F-actin fragmenting protein	35,25	8,64
SmlA	DDB0191525	SMaLl aggregates	33,15	6,07
TubA	DDB0191380	alpha tubulin	50,93	5,34
TubB*	DDB0191169	beta tubulin	51,30	5,12
Intracellular protein transport (4)				
BC5V2_0_00780	DDB0188107	similar to vacuolar protein sorting 29	20,40	6,17
BC5V2_0_01487	DDB0188782	vacuolar protein sorting-associated protein 45	64,53	5,88
DDB0189960	DDB0189960	coatomer delta subunit	60,30	5,61
PrtC	DDB0214956	proteasome subunit C2 homolog	27,97	6
Stress response (13)				
Response to stress				
Aif	DDB0191137	apoptosis inducing factor	59,63	6,07
PefA	DDB0191092	penta EF hand calcium binding protein	22,34	7,14
Response to oxidative stress				
CatA	DDB0185123	Catalase	55,64	8,61
BEC6V2_0_00593	DDB0184362	stress-induced sti1-like protein	63,17	6,16
Response to osmotic stress				
BC4V2_0_00670	DDB0185918	Protein phosphatase 2C homolog 1	44,39	4,98

Response to drugs (3)				
MvpA*	DDB0191259	major vault protein	94,07	6,05
MvpB	DDB0191337	major vault protein B	95,01	5,61
SksA	DDB0191298	sks1 multidrug resistance protein homolog	34,43	6,07
Heat shock proteins (5)				
BC4V2_0_01427	DDB0186670	hsp-70-related intracellular vitamin D binding	57,33	5,89
HspB	DDB0191168	heat shock cognate protein Hsc70-1	70,37	5,34
HspD	DDB0191163	heat shock cognate protein	79,82	5,02
HspE*	DDB0185047	heat shock protein	69,75	5,47
JC2V2_0_01264	DDB0217225	heat shock protein 70	69,75	5,47
mRNA splicing & regulation of transcription (2)				
JC3V2_0_00295	DDB0205391	nucleic acid binding (IEA)	36,56	9,98
JC2V2_0_02739	DDB0167100	transcription factor activity	40,02	6,99
ER (4)				
BC5V2_0_00839	DDB0188166	endosperm C-24 sterol methyltransferase	39,53	6,16
DDB0231409*	DDB0231409	protein disulfide isomerase	57,72	5,17
JC2V2_0_02727	DDB0167089	glucose-regulated protein homolog precursor	72,49	5,13
JC3V2_0_01253	DDB0206509	integral to membrane	19,64	5,25
Iron transport (4)				
BC4V2_0_01063	DDB0186311	hemopexin domain signature	31,67	4,8
BEC6V2_0_01236	DDB0219884	hemopexin domain signature	80,34	5,19
FhbA	DDB0191099	Flavohemoglobin	43,90	6,88
FhbB	DDB0191088	Flavohemoglobin	48,18	5,16
Unknown function (15)				
BC4V2_0_00175	DDB0218473	Unknown	45,29	6,19
BC4V2_0_00250	DDB0218489	Unknown	26,24	5,27
BC5V2_0_00316	DDB0219276	Unknown	37,50	5,17
BC5V2_0_00460	DDB0187793	Unknown	29,08	9,54
BC5V2_0_01113	DDB0188434	Unknown	75,20	6,32
BC5V2_0_01650	DDB0188942	Unknown	53,33	6,06
CinB	DDB0220110	Unknown	38,53	5,61
DDB0229909*	DDB0229909	Unknown	25,84	5,57
JC1V2_0_00135	DDB0189346	Unknown	39,08	5,4
JC1V2_0_00564	DDB0189754	Unknown	24,53	6,71
JC1V2_0_00789	DDB0202301	Unknown	33,78	8,28
JC2V2_0_00793	DDB0168923	dihydropteridine reductase domain signature	24,65	6,9
JC2V2_0_01572	DDB0217484	Unknown	99,02	4,98
JC3V2_0_00478	DDB0206195	Unknown	25,97	7,6
JC3V2_0_01972	DDB0204655	Unknown	40,32	8,55

* The proteins significantly changed between *L. pneumophila* Corby and *L. hackeliae*-specific phagosome are marked by asterisk.

Proteins that are likely to be associated with the phagosome membrane and vesicular transport were also identified. These include minor amounts of two subunits of the vacuolar ATPase (V-type H+-ATPase subunits A and B), which is a multisubunit complex that functions to generate and maintain the acidic environment of the phagolysosome. Other groups belong to the mitochondrial class (10 proteins), intracellular protein transport and ER category (8 proteins), oxidoreductase activity category (5 proteins), vacuolar H+ ATPase category (5 proteins) and transcription regulation and mRNA splicing (2 proteins). Taken together, we have identified many proteins with an evident role in phagocytosis or phagosome maturation. In addition we found several proteins for which a link to phagosomal processes remains to be established. The temporal and pathogenic changes of the phagosomal proteome will be described in the following.

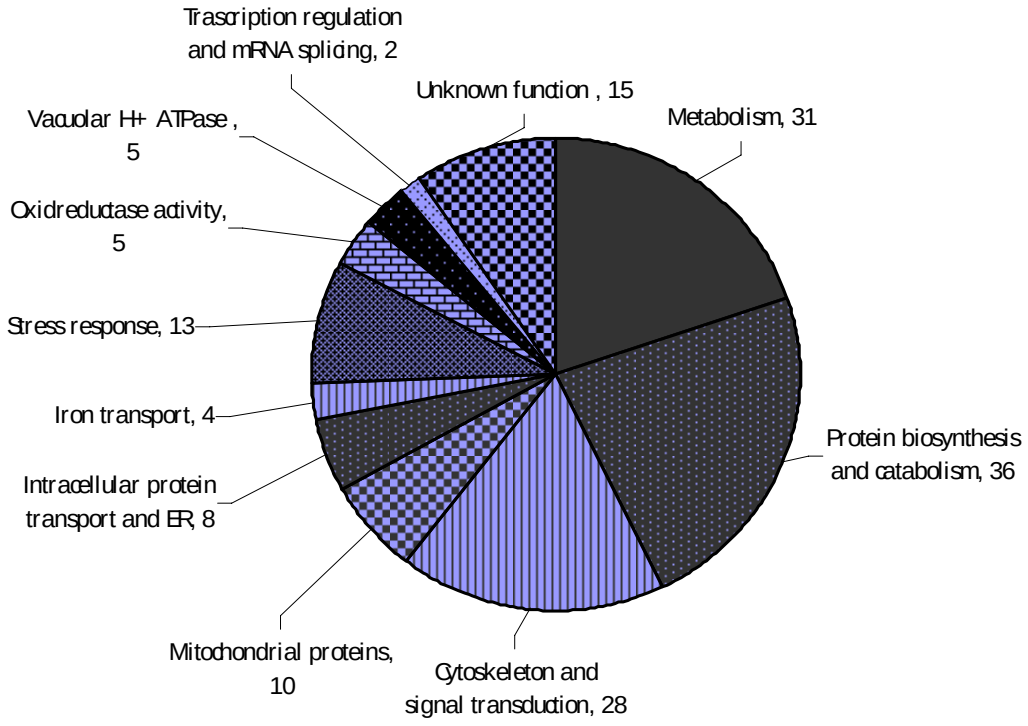


Fig. 5-11 Functional categories of the identified proteins
The phagosomal proteome was categorized based on gene ontology annotations in *D. discoideum* database (http://www.dictybase.org). The total number of proteins (n=157) was divided into eleven functional categories based on biological process or molecular function of every protein: metabolism (31), protein biosynthesis and catabolism (36), cytoskeleton and signal transduction (28), mitochondrial proteins (10), proteins implicated in intracellular transport and belong to endoplasmatic reticulum (8), iron transport (4), stress response proteins (13), proteins with oxidoreductase activity (5), subunits of H+ ATPase, proteins involved in transcription regulation and splicing (2), proteins without known or predicted function (15). The number of proteins in each category is indicated by braces.

5.2.2.2 Identification of variably expressed phagosomal proteins during infection with *L. pneumophila* Corby or *L. hackeliae*

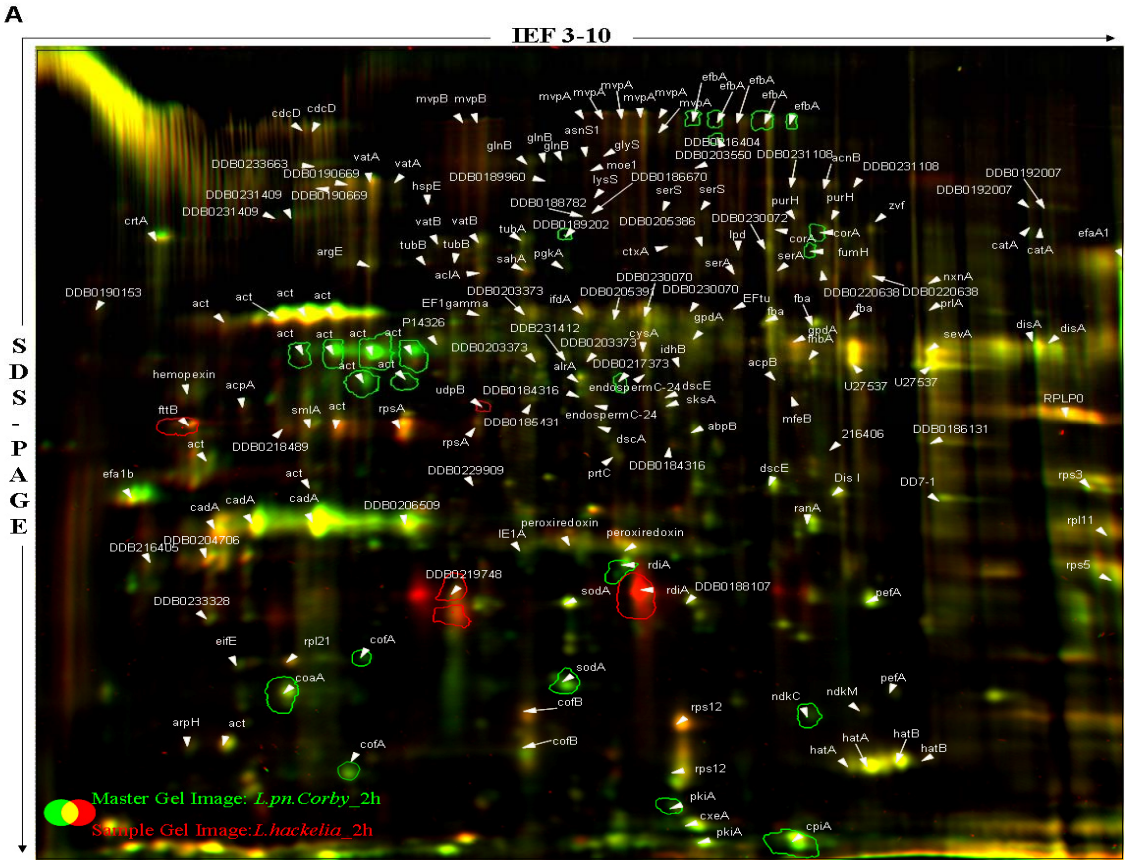
Different *Legionella* species display different behaviour within host cells. Well characterized and sequenced strains, *L. pneumophila* Corby and *L. hackeliae* were choosen for analysis. Both strains are taken up by phagocytosis. However, only *L. pneumophila* Corby is capable of growing in host vacuoles which are protected from lysosomal degradation. At the same time *L. hackeliae* fails in acidification arrest, reduced pathogenicity in macrophages and is degraded by *D. discoideum* (Hägele et al., 2000).

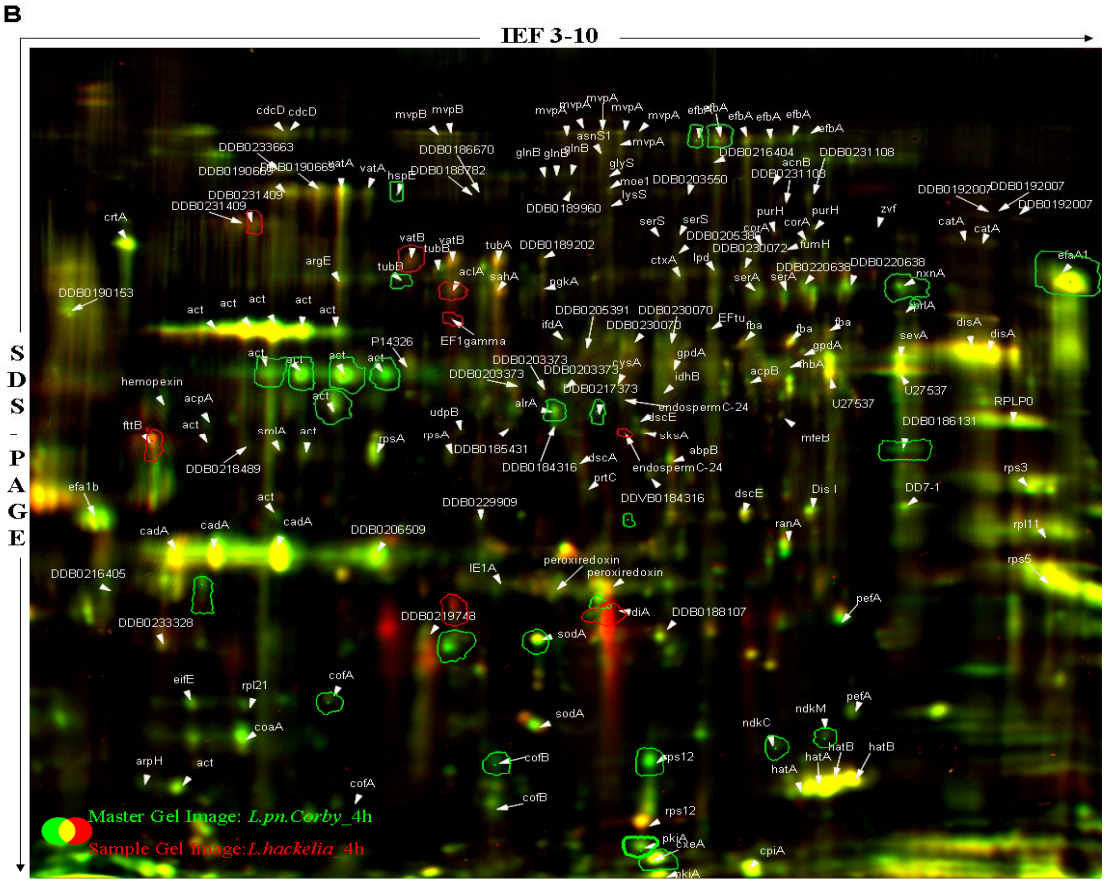
To elaborate the differences between *L. pneumophila* Corby- and *L. hackeliae*-infected phagosomes, the respective phagosomal proteins were systematically identified at 2, 4 and 6 hours post infection. In order to achieve high accuracy two or three independent isolation experiments with equal amounts of phagosomal proteins were used for each time point and the resulting fluorescence stained 2D gels were analyzed with the Delta2D software program (Decodon, Germany). The analysis protocol included spot detection and filtering, whole image warping on a reference gel, background subtraction and average gel creation.

Only changes in spot representation of 2.0 or higher were analyzed. Figure 5-12 A-C presents a gallery of dual colour 2-DE images (green fluorescent dye Cy3 for *L. pneumophila* Corby and red fluorescent dye Cy5 for *L. hackeliae*) exhibiting the differences between *L. pneumophila* Corby and *L. hackeliae* phagosomes.

Together, these data illustrate slight differences between the proteomic profiles of the phagosome of these two *Legionella* species. 22, 25 and 26 differentially regulated spots were identified for 2, 4 and 6 hours phagosome, respectively (Table 5-3). Interestingly, *L. pneumophila* Corby phagosome -associated proteins are generally upregulated to a greater extent (2.2- to 10.0-fold) than those in *L. hackeliae* strain.

A significant increase in protein amount was observed in the case of cystein proteinase inhibitor, and one of the spots representing Rho GDP-dissociation inhibitor in *L. pneumophila* Corby phagosome at 2 hours after infection. These proteins are known to be involved in different signalling pathways. At the same time *L. hackeliae* phagosomes were enriched by elongation factor 1 alpha, uridine phosphorylase and polypeptide with oxidoreductase activity (Table 5-3).


A
IEF 3-10
SDS-PAGE
Master Gel Image: L.pn.Corby_2h
Sample Gel Image:L.hackelia_2h


B
IEF 3-10
SDS-PAGE
Master Gel Image: L.pn.Corby_4h
Sample Gel Image:L.hackelia_4h

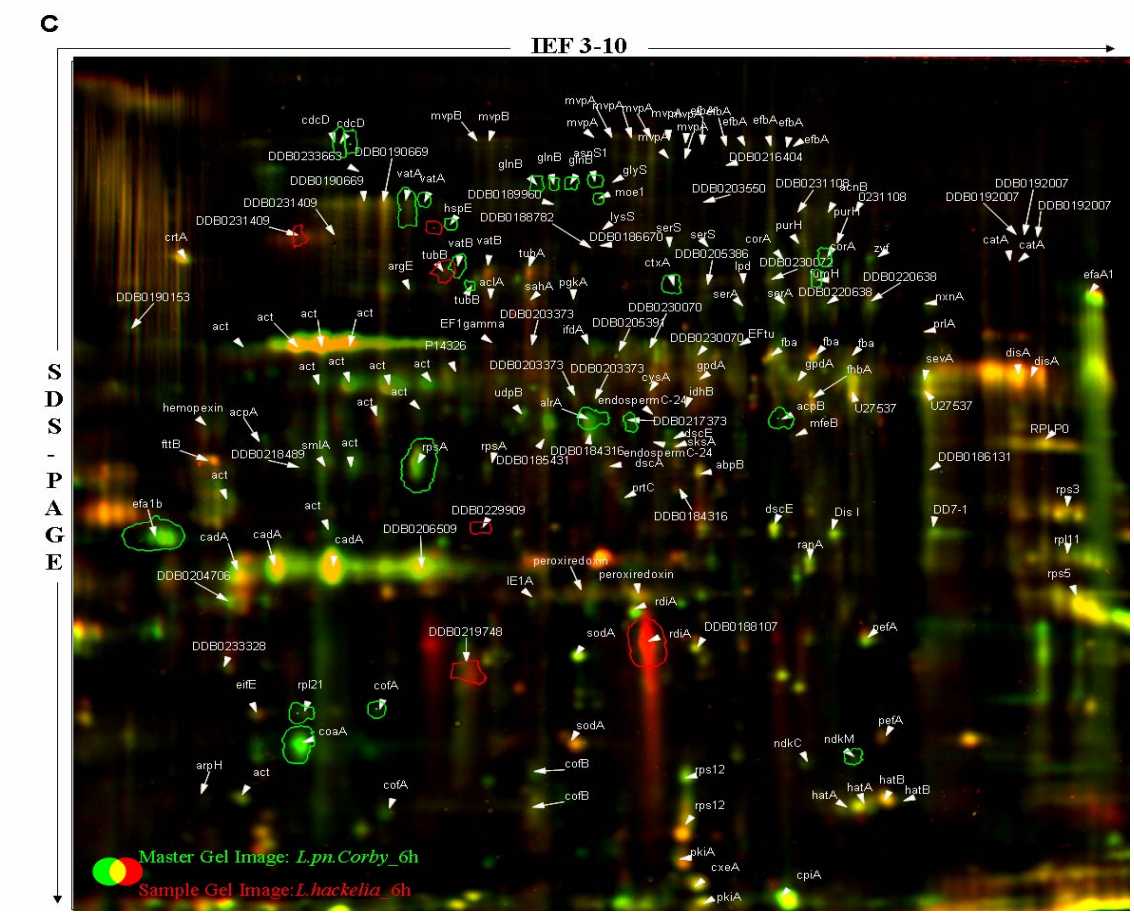


Fig. 5-12 Dual channel images of the phagosomal protein patterns
A. Pattern 2 hours post infection.
B. Pattern 4 hours post infection.
C. Pattern 6 hours post infection.
Phagosome proteins from *D. discoideum* cells infected with *L. pneumophila* Corby were labelled with Cy3 (green) while phagosome proteins from *L. hackeliae* infections were labelled with Cy5 (red). All proteins differentially expressed are highlighted and listed in table 1 and 2. The % volume in table 2 indicates the relative quantity of the spot, excluding background. The signal intensity of all spots on the gel is 100 %. The ratio represents a fold change of protein expression (*L. pneumophila* Corby phagosome/*L. hackeliae* phagosome).

At all observed time points of infection the Rho GDP-dissociation inhibitor was represented by two spots with slightly different molecular weight in phagosomal proteome pattern. One of these spots is upregulated in *L. pneumophila* Corby phagosome, the other in *L. hackeliae* phagosomes (Table 5-3).

At 4 hours after infection enrichment by annexin VII (ANXA7; ANX7; synexin) was observed in *L. pneumophila* Corby phagosomes in contrast to *L. hackeliae* phagosomes. Annexin belongs to the family of phospholipid binding proteins.

The molecules belonging to the protein biosynthesis class (*efa1b*, *moe1*, ribosomal proteins and asparaginyl-tRNA synthetase) appear at 6 hours post infection in pathogenic *Legionella*-phagosomes. The same is true for actin binding proteins like AcpA, AcpB, cortexillin and coronin.

Table 5-3 List of phagosomal proteins significantly modulated during infection with different *Legionella* species. The % volume in the table indicates the relative quantity of the spot, after subtraction of the background. The intensity of all spots on the gel is 100 %. The ratio represents a fold change of protein expression (*L. pneumophila* Corby phagosome/*L. hackeliae* phagosome)

Protein	Function	% volume; *L. pneumophila* Corby	% volume; *L. hackeliae*	Fold change
***L. pneumophila* Corby / *L. hackeliae* 2 hours**				
CpiA	cysteine proteinase inhibitor	0,99	0,09	10.0
CofA	cofilin	0,11	0,01	10.0
DDB0189202	peptidase M16 family protein	0,04	0,01	5.3
Act	actin	0,93	0,19	5.0
Act	actin	0,35	0,09	3.7
CorA	coronin	0,17	0,05	3.6
SodA	superoxide dismutase	0,68	0,19	3.6
Act	actin	0,64	0,19	3.4
Act	actin	0,16	0,05	3.2
Act	actin	0,97	0,32	3.0
Act	actin	2.01	0,71	2.8
NdkC	nucleoside diphosphate kinase	0,19	0,07	2.8
DDB0217373	similar to discoidin I, A chain	0,08	0,03	2.7
FumH	fumarate hydratase	0,13	0,05	2.4
PkiA	similar to protein kinase C inhibitor	0,24	0,10	2.2
RdiA	Rho GDP-dissociation inhibitor	0,54	0,25	2.1
EfbA	elongation factor 2	0,09	0,04	2.1
EfaA1	elongation factor 1 alpha	1,29	3.06	0.4
FttB	14-3-3 protein	0,17	0,49	0.3
DDB0219748	oxidoreductase activity	0,36	1,10	0.3
UdpB	uridine phosphorylase	0,01	0,04	0.1
RdiA	Rho GDP-dissociation inhibitor	0.56	4.42	0.1
***L. pneumophila* Corby / *L. hackeliae* 4 hours**				
CxeA	cytochrome c oxidase subunit V	1.00	0.45	2.2
PkiA	similar to protein kinase C inhibitor	0,23	0,11	2.2
HspE-1	heat shock protein	0,004	0,0001	2.5
Rps12	ribosomal Protein Small subunit	0,73	0,14	5.3
Act	actin	0,37	0,07	5.0
DDB0217373	similar to discoidin I, A chain	0,07	0,01	5.0
CofB	cofilin	0,37	0,10	3.8
Act	actin	1,00	0,30	3.4
Act	actin	0,90	0,29	3.1
AxnA	annexin VII	0,32	0,13	2.4
Act	actin	0,14	0,06	2.3
NdkC	nucleoside diphosphate kinase	0,11	0,05	2.2
TubB	tubulin	0,04	0,02	2.2
AlrA	aldehyde reductase	0,42	0,19	2.2
NdkM	nucleoside diphosphate kinase	0,11	0,05	2.1
PrsA	phosphoribosyl pyrophosphate synthetase	0,63	0,30	2.1
Act	actin	1,67	0,83	2.0
VatB	vacuolar H+ ATPase B subunit	0,08	0,16	0.5
MvpA	major vault protein A	0,05	0,10	0.5
Pdi2	protein disulfide isomerase	0,10	0,21	0.5

AclA	component of Arp2/3 complex	0,12	0,26	0.4
FttB	14-3-3 protein	0,43	0,98	0.4
DDB0188166	putative delta24sterol methyltransferase	0,02	0,04	0.4
RdiA	Rho GDP-dissociation inhibitor	0.83	5.26	0.2
***L. pneumophila* Corby / *L. hackeliae* 6 hours**				
GlnB	glutamate-ammonia ligase	0,03	0,001	25.0
AcpB	actin capping protein	0,06	0,006	10.0
CofA	cofilin	0,09	0,02	5.9
CdcD	cell division cycle protein 48	0,07	0,02	4.5
CoaA	coactosin	1.69	0,38	4.5
NdkM	nucleoside diphosphate kinase	0,12	0,03	4.5
Moe1	eIF-3, subunit 2	0,02	0,004	4.0
AspS1	aspartyl-tRNA synthetase	0,02	0,005	3.3
Act	actin	0,19	0,06	3.3
CtxA	cortexillin I	0,05	0,02	2.9
VatA	vacuolar H+ ATPase B subunit	0,25	0,09	2.9
DDB0217373	similar to discoidin I, A chain	0,18	0,07	2.6
Act	actin	0,74	0,28	2.6
AlrA	aldehyde reductase	0,68	0,26	2.6
Efa1b	elongation factor 1 beta	1,97	0,78	2.6
AcpA	actin capping protein	0,09	0,04	2.4
GlnB	glutamate-ammonia ligase	0,06	0,03	2.3
DscE	discoidin II	0,14	0,06	2.3
CorA	coronin	0,16	0,07	2.2
Rpl21	ribosomal protein L11	0,27	0,12	2.2
RpsA	40S ribosomal protein SA	1,24	0,57	2.2
DDB0219748	oxidoreductase activity	0,36	0,80	0.4
DDB0229909	unknown function	0,002	0,01	0.2
RdiA	Rho GDP-dissociation inhibitor	0.73	5.67	0.1
TubB	tubulin	-	0,02	∞
Pdi2	protein disulfide isomerase	-	0,24	∞

In summary, the prominent changes in protein composition are related to the recruitment of factors involved in protein biosynthesis and proteins implicated in cytoskeleton organisation and rearrangement by *L. pneumophila* Corby. At the same time *L .hackeliae* phagosome acquires Rho GDP-dissociation inhibitor and FttB, a protein which has apparently diacylglycerol-activated phospholipid-dependent protein kinase C inhibitor activity.

5.2.2.3 Actin degradation caused by *Legionella* infection.

Actin is essential for many cellular processes, including phagocytosis and membrane fusion (Kjeken et al. 2004). In *Legionella*-specific phagosomes actin is represented by numerous spots on the proteome map. At all stages during infection by *L. pneumophila* Corby versus *L. hackeliae,* the appearance of additional actin spots, which migrated faster than 40 kDa actin in 2D SDS-PAGE was detected (Fig.5-13a). To test whether these degradation products are present in the *Legionella* phagosomal fraction a specific *D. discoideum* antibody against actin was used. Western blot analysis revealed an additional actin band about of 36 kDa in size only in the case of *L. pneumophila* Corby infection (Fig.5-13b).

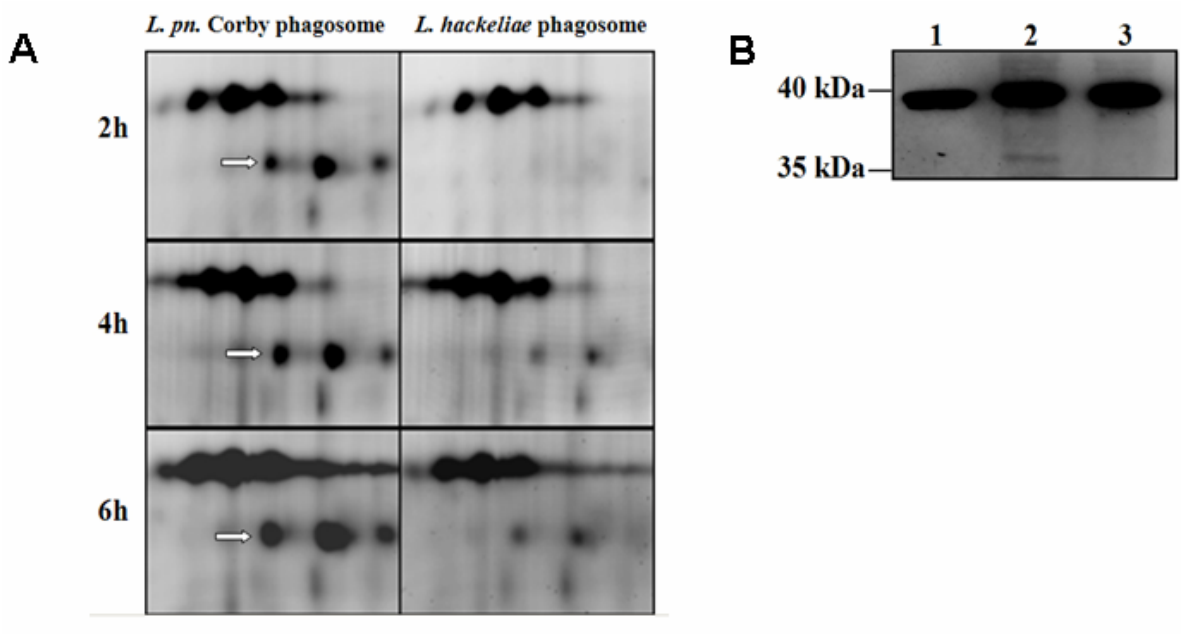


Fig. 5-13 Actin degradation caused by *L. pneumophila* Corby infection.
A. Actin spots on the 2D gels from *L. pneumophila* Corby phagosome (left panel) and *L. hackeliae* phagosome (right panel). Arrows indicate additional spots, which migrated faster than undegraded actin.
B. Western blot analysis of 1D SDS-PAGE demonstrated the appearance of degraded actin. Lane 1: Isolated *D. discoideum* actin (control). Lane 2: *L. pneumophila* Corby phagosome fraction. Lane 3: *L. hackeliae* phagosome fraction. Proteins were separated on SDS-polyacrylamide gel, blotted onto a nitrocellulose membrane and incubated with a specific anti-actin-1 antibody.

5.2.2.4 Analysis of temporal changes of the phagosomal proteome during infection with *L. pneumophila* Corby or *L. hackeliae*

The *Legionella* are taken up into phagosomes, which the bacteria modify to provide an environment favorable for replication. The main goal of this work was the analysis of species-specific phagosomal modulation. Fig. 8-2 presents a gallery of fluorescent 2D gels of phagosomes extracted at three time points of infection. Figures 8-2A and 8-2B represent the comparisons of 2 hours/4 hours and 4 hours/6 hours of *L. pneumophila* Corby infection, respectively. Figures 8-2C and 8-2D represent the comparisons of 2 hours/4 hours and 4 hours/6 hours of *L. hackeliae* infection, respectively. Significantly modulated proteins are circled.

Table 5-4 *L. pneumophila* Corby phagosomal proteome alteration

Functional classification	Name of gene	2 hours/4 hours			4 hours/6 hours		
		# spots	# regulated spots	ration % volume	# spots	# regulated spots	ration % volume
Protein metabolism	*serS*				2	1	3,49
	efbA°	5	1	0,43			
	eifE	1	1	2,9			
	RPLP0	1	1	0,44			
Protein folding and degradation	*prlA*	1	1	0,34			
	disA	2	1	4,71			
		2	1	2,25			
Amino acid metabolism	*DDB0230070*						
Carbohydrates metabolism	*Fba*	2	1	0,45			
	DDB0231108				3	1	2,46
Cytoskeleton and signal transduction	*arpH*				1	1	0,4
	cdcD				2	1	0,4
	crtA	1	1	2,2			
Stress response	*pefA**	2	1	2,03			
	hatA°				2	2	0.34;0.3
	DDB0186670				1	1	4
	mfeB				1	1	0.41
							6.11
V-ATPase and ATP synthase	*vatA*	2	1	5,86			
	vatB	2	1	0,42			
	DDB0184316	1	1	2,37	1	1	6,11
	DDB0190669	2	1	2,57			2,27

Table 5-5 *L. hackelia* phagosomal proteome alteration

Functional classification	Name of gene	2 hours/4 hours			4 hours/6 hours		
		# spots	# regulated spots	ration % volume	# spots	# regulated spots	ration % volume
Protein metabolism	*rps12*	2	1	0,13	2	1	3,13
	rpl21	1	1	0,35			
	rpsA	2	2	0,38; 0,49			
	efaA1	1	1	0,22			
	efbA˚				5	2	0,11
	efbA˚				5	2	0,27
	efa1b				1	1	0,38
	eifE				1	1	2,18
	ifdA	1	1	0,45			
Purine & pyrimidine metabolism	*disA*	2	2	2,23; 2,33			
Carbohydrates metabolism	*udfB*	1	1	0,17			
Cytosceleton and signal transduction	*fba*	3	1	2,51			
Stress response	*cofB*	2	1	0,23			
	acpA	1	1	0,24			
	nxnA	1	1	0,4			
	arpH	1	1	0,42			
	fttB	1	1	2,11			
	cofA˚	2	1	2,39	2	1	0,4
	cdcD				2	2	0,14; 0,28
	pgkA				1	1	0,36
	pkiA				1	1	2,39
	sodA	2	1	2,26			
	hatA˚	2	1	2,07	2	2	0,38; 0,4
	hatB				2	1	0,24
	mvpA				7	1	0,24
Unknown function	*DDB0229909*	1	1	0,23			
	DDB0203550	1	1	0,25			
	DDB0218489	1	1	0,34			
	DDB0231409	1	1	2,34			
	DDB0190153				1	1	0,44

To simplify the analysis of the phagosome maturation process we have divided the proteins with expressional changes into functional classes. Polypeptides, represented by several spots, probably indicated the modification or degradation of the protein. The comparisons of the two hour time point with the four hour time point and the four hour time point with the six hour time point of *L. pneumophila* Corby and *L. hackeliae* are shown in Table 5-4 and Table 5-5, respectively.

In summary, the data indicated distinct phases of the phagosomal maturation process, which corresponded to specific protein up- and down regulations and protein modifications rather than the appearance or disappearance of host proteins. More specifically, a significant decline in quantity of actin binding proteins (CofB, AcpA, NxnA, ArpH) and proteins involved in biosynthesis (Rps12, Rpl21, RpsA, EfaA1, IfdA) was observed at four hours in the case of *L. hackeliae* infections. In contrast *L. pneumophila* Corby phagosomes acquire calreticulin and subunits of V-ATPase. Interestingly, stress response proteins show decreased expression in both cases at six hours post infection. We observed disappearance of HatA and DDB0186670 in *L. pneumophila* Corby phagosomes and HatA, HatB and MvpA in *L. hackeliae* phagosomes.

5.2.2.5 Modification of *Legionella* phagosomal proteins

Reversible phosphorylation is considered to be one of the important modifications regulating the functional status of proteins. Therefore, the phosphorylation status of phagosomal proteins was checked combining the Pro-Q Diamond staining with MS. By this sensitive noncovalent fluorescent dye staining technology for the detection of phosphoserine-, phosphothreonine- and phosphotyrosine-containing proteins we proved phosphorylation of the 4 proteins (Fig. 5-14). These were eukaryotic elongation factor 2 (EfbA), ribosomal acidic phosphoprotein (PORs), elongation factor1δ and translation initiation factor (EifE).

All four phosphorylated proteins are involved in the protein biosynthesis. PORs - ribosomal acidic phosphoprotein is apriori phosphorylated. EfbA (eEF2) appears on the 2D gels as row of spots, shifting in pH, probably caused by phosphorylation of several amino acids. This factor mediates ribosomal translocation, and its activity is inhibited by phosphorylation. The kinase acting on eEF2 depends on calcium ions and calmodulin. Recently, it was reported that a slight decrease in pH, within the range observed *in vivo*, leads to dramatic activation of this kinase followed by inhibition of protein biosynthesis (Browne & Pround 2002, Dorovkov, Pavur, 2002). Unfortunately it was not possible to identify the modified amino acids by MS/MS. Further study on phosphorylation of these proteins must include an additional phosphopeptide enrichment step.

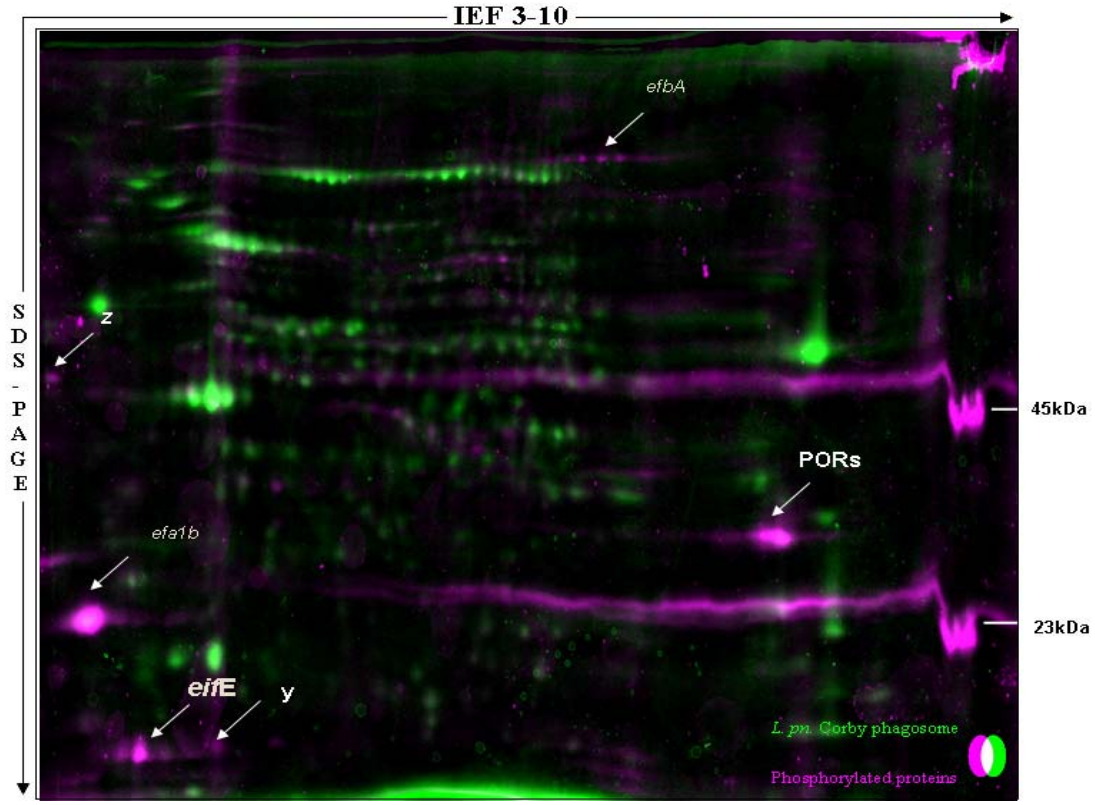


Fig. 5-14 Visualization of total protein (green) and phosphoproteins (pink) in a single 2-D gel
Phagosomal proteins isolated from *D.discoideum* infected by *L. pneumophila* Corby after 4 hours of infection were separated by 2-D gel electrophoresis and stained with Pro-Q Diamond phosphoprotein gel stain (pink).

6 Discussion

6.1 *D. discoideum* as a screening system for bacterial virulence estimation and searching for new virulence determinants

Since *D. discoideum discoideum* was established as a suitable organism to study host-pathogen interaction, the number of researchers who have utilized this amoeba as a model system has increased (Hagele et al. 2000; Solomon et al. 2000; Steinert and Heuner 2005). It is especially attractive to study intracellular pathogens, as many basic aspects of *D. discoideum* signal transduction, cell motility and phagocytosis are similar to the respective processes in mammalian professional phagocytes.

Two screening systems were represented in this work:

The first one was a simple screen (plaque assay) for determination of bacterial virulence potential based on *D. discoideum* predation properties. This screen allowed us to analyse different bacterial strains or pathogens attenuated by mutagenesis for their virulence potential. Recently this method was used for determination of avirulent mutants in *Pseudomonas aeruginosa* as well as for the extracellular pathogen *Vibrio cholerae* (Pukatzki et al. 2002; Pukatzki et al. 2006). These microorganisms use different strategies to kill *D. discoideum* cells. The former utilizes T3SS to deliver cytotoxic proteins ExoU, encoding phospholipase A, inside host cells, whereas the second one prevents plaque formation by secreting contact dependent cytotoxins.

With help of the the plaque assay 12 *Legionella* species were analyzed, including well-characterized sequenced strains, patient isolates and *Legionella*-like amoebal pathogens. According to the plaque assay data, the analyzed strains can be divided into three classes (with high, medium and low virulence). This classification is in accordance with the already described characteristics of the strains. This allows us to conclude that this screening is useful to determine the virulence potential of *Legionella* strains as well as for pre-screening of strains altered in virulence due to mutagenesis.

The second screening represented a method for isolation of *Legionella* mutants defective in the reprogramming of phagolysosomal maturation of the host. We have applied a previously described method used for isolation *Mycobacterium tuberculosis* mutants defective in control of phagosomal acidification to a *Legionella- Dictyostelium* host interaction model (Pethe et al. 2004).

Legionella is known to survive and replicate within the host phagosome. The comparison of infected macrophages and *D. discoideum* cells demonstrated that in both host systems virulent *L. pneumophila* phagosomes were associated with the rough endoplasmic reticulum. During early stage of infection the *Legionella*-containing phagosomes exclude endocytic and lysosomal markers,

which is recognized as a key feature of *Legionella* pathogenicity. These *Legionella* properties were used to evaluate a mechanical screening to obtain a set of mutants, defective in host cell phagosomal maturation. After four rounds of selection it was possible to isolate the mutants with identical genotype, unable to prevent phagosomal acidification. Obtained mutants are potentially involved in manipulation of host signalling pathways by which *Legionella* avoid phagolysosomal degradation.

This method is presumably applicable for other intracellular pathogens that block the progression of the phagosome maturation, survive transient exposure to lysosomal contents and allow for a feasible mutational approach.

6.2 Application of the array technology for determination of *Legionella* virulence factors

DNA microarray technology plays an important role in modern infection disease research. The completion of numerous of pathogen genome sequencing and description of main virulence factors allowed us to generate an array with a set of the most important virulence determinants of bacterial pathogens. Additionally, the most common selection markers and antibiotic resistance genes for detection of genetically or naturally modified microorganisms were added to the array.

In this study the array was used for detection of virulence genes in twelve *Legionella* strains. As expected, the more virulent bacteria possess a higher number of pathogenicity determinants. We have identified *mip* (macrophage infectivity potentiator), encoding an important component of *Legionella* pathogenesis, in all *L. pneumophila* strains. Moreover *L. micdadei*, *L. hackeliae* and LLAP10 possess *mip*-like genes. Nucleotide sequence analyses of these genes reveal 72.1 %, 70.9 % and 80.9 % homology to *L. pneumophila* Corby *mip*, respectively. Whether all strains analysed produce a functionally active Mip protein is still unknown. Previously, Cianciotto and colleagues suggested, that Mip and Mip-like proteins play a significant role in resistance of *Legionella* strains to intracellular killing (Cianciotto et al. 1990). *L. hackeliae* is not known to be able to survive during *D. discoideum* infection, although it possesses a *mip*-like gene. Therefore, it was proposed that this protein may play another role in infection. Recently it was shown that the Mip protein has a collagen binding activity. This activity together with the serine protease activity allows *Legionella* to penetrate the lung epithelial barrier and therefore disseminate the bacteria within the lung tissue (Wagner et al. 2007).

Other potential virulence factors, previously not identified in non-*pneumophila Legionella* strains are phospholipases. Phospholipase-like activities are conserved among clinical isolates of *L. pneumophila* as well as other pathogenic *Legionella* species (Baine 1985).

We have detected the *plcA* gene, encoding a putative phospholipase C (PLC) among *L. pneumophila* and one non-*pneumophila* strain, *L. longbeacheae*. The PlcA protein is highly

homologus to a new type of PLC that was found in *Pseudomonas fluorescens* (Aragon et al. 2002). Whether the non-*pneumophila* strains, which possess a *plcA*-like gene encode a functional phospholipase C protein is not known and needs to be tested. Interestingly, Mip contributes to the presence of phospholipase C-like activity in culture supernatants (Debroy et al. 2006).

The cell-associated phospholipase PlaB contributes to bacterial cytotoxicity due to its haemolytic activity (Flieger et al. 2004). Considering that this enzyme possesses a high impact on virulence it was expected that *plaB* would be present in *L. pneumophila* species and absent in non-*pneumophila* strains. Nevertheless, Southern blot hybridization and biochemical enzyme tests revealed that the two non-*pneumophila* strains, *L. spiritensis* and *L. gormanii*, do possess *the plaB* gene or *plaB*-like gene and cell-associated PLA and LPLA activities (Heuner 2007).

By array analysis four additional non-*pneumophila* strains possessing phospholipase-like genes *L. erytra, L. bozemanii*, *L. hackeliae* and LLAP10 were identified. *L. hackeliae* and LLAP10 showed 83.2 % and 83.8 % homology to *L. pneumophila* Corby *plaB*, respectively (*L. erytra, L. bozemanii plaB* genes are not sequencesed, and therefore not analysed). The membrane fraction of *L. hackeliae* possesses low haemolytic activity compared to *L. pneumophila* Corby, while *L. erytra, L. bozemanii* and LLAP10 do not exhibit any haemolysis. However, we can not exclude that hemolysis in *L. hackeliae* was caused by other enzymes, which are not yet identified.

At the moment seven *Legionella* genomes have been sequenced. The four strains *L. pneumophila* Corby, *L. pneumophila* Paris, *L. pneumophila* Lens and *L. pneumophila* Philadelphia1 caused the majority of Legionnaire's disease, and all are annotated and presented in the PubMed database. The other three strains *L. micdadei*, *L. hackeliae* and LLAP10 are being analyzed. In general, it is possible to say that more pathogenic strains possess more virulence determinants. However, we should keep in mind that presence of individual virulence genes has not always correlated with pathogenic phenotype of bacteria.

In summary, with the help of the DNA microarray technology the differences in virulence potential between *Legionella* species have been demonstrated. The sequences of a great number of strains associated with disease are not available, therfore the array can be a powerful tool for simultaneous detections of virulence factors. This array could be also helpful for potential risk assignment of water as well as for other environmental samples.

6.3 Isolation of *Legionella*-containing phagosomes

The first mentioned phagosomal isolation was performed for *Chlamydia psittaci* infected L cells (Zeichner 1982). A number of studies, which improved the isolation protocol, are available (Luhrmann and Haas 2000; Ramachandra et al. 1998; Sturgill-Koszycki et al. 1997). Most of them described the preparation of the phagosome from the infected macrophages and are based on the separation of the intracellular organelle by a discontinuous gradient centrifugation. The great

progress in this field and better understanding of the phagocytosis event have all resulted from the molecular characterisation of the latex beads phagosome (Desjardins et al. 1994; Garin et al. 2001). Using the proteome approach more than 200 different proteins were detected and their evidence in phagocytosis was suggested. Moreover, a technique for preparation of the highly purified *D. discoideum* latex beads phagosomal fraction was established last year. Based on this technique, the proteome composition and their alteration in time were analysed (Gotthardt et al. 2006a; Gotthardt et al. 2006b).

This work is the first description of a method for the isolation of *Legionella*-containing phagosomes. The intracellular behavior of the pathogenic phagosomes, including fusion with other organelles, recruitment of various proteins from the cytoplasm and representation of additional bacterial peptides, is proposed to be dependent on their lipid and protein membrane composition (Desjardins 1995). Previously, the different aspects of the *Legionella* phagosome maturation process has been studied on the cellular level by using electron microscopy methods, host cell mutants, GFP-tagged host proteins and fluorescence-labelled monoclonal antibodies. Despite this, little is known about the exact protein phagosomal composition. The work herein represents for the first time the phagosomal isolation method optimized by: treatment with Benzonase, loading the endocytic compartments with colloidal iron and increasing the density of mitochondria by substrate specific reaction.

The Benzonase treatment of postnuclear supernatant removes nucleic acid and decreases viscosity of the intracellular soup. This facilitates the organelle separation and results in a better spot resolution in 2D gel electrophoresis. Colloidal iron particles load the endocytic compartment and lysosomes allowing removal of these organelles by a magnet. Finally, the mitochondria fraction and bacteria-phagosome fraction could not be clearly separated by a gradient centrifugation. The phagosomes were located in fractions with a density of 1.16–1.19 g/ml (Via et al. 1997). The density of mitochondria in sucrose gradients is about 1.17-1.18 g/ml. The treatment of cell homogenate with a tetrazolium dye slightly increased the mitochondria density (1.21 g/ml) and at the same time left both organelles intact (Parish 1975).

The purity of the isolated organelles is very important for the following proteome analyses. We therefore evaluated it by several methods, including immunoblot for specific organelle markers and transmission electron microscopy of the fractions. For immunoblot ER resident protein- Calnexin was chosen because in previous studies the accumulation of this marker in the phagocytic cup of *Legionella*-infected *D. discoideum* cells was shown. Moreover, a double mutant for the calnexin and another Ca^{2+} binding ER protein calreticulin completely abolished phagocytosis in *D. discoideum* (Fajardo et al. 2004; Muller-Taubenberger et al. 2001).

The calnexin localised in ER displays three domains: a signal sequence followed by a (1) N-terminal luminal domain, (2) a single transmembrane domain and (3) a C-terminal cytoplasmic

domain. Interestingly, using specific calnexin recognizing antibodies we observed two bands, one which was about 80 kDa and another about 60 kDa, while the calculated molecular weight of calnexin is 60,5 kDa. A similar phenomenon was mentioned previously with neutrophil phagosomes. While the intact calnexin observed at around 95 kDa by gel electrophoresis was present on early phagosomes, its degraded forms were observed in maturing phagosomes. Because the C-terminal region of the calnexin present in phagosomes was sensitive to the protease treatment, the authors suggested that this cleavage occurs in the phagosome lumen by proteases acquired during phagolysosome biogenesis (Gagnon et al. 2002).

In summary, we were able to establish a method for isolation of a phagosome enriched fraction. The purity of phagosome was confirmed with immunological and morphological methods.

The study of the isolated organelles could be pursued in several directions. First, the present isolation procedure is suitable for proteome analysis of this organelle. A detailed analysis of the phagosomal proteome composition allows a global view of potential functions of these organelles. Second, a modification of phagosomal proteins, like phosphorylation or glycosylation, could also be investigated. Finally, the proteome pattern of phagosome permits the monitoring of the kinetics of infection process.

6.4 Protein composition of *Legionella*-containing phagosome

The determination of the phagosomal protein composition allowed the generation of a phagosomal proteome map. This proteome map has been used for analyzing protein patterns during infection and between *L. pneumophila* Corby and *L. hackeliae* species. Moreover, the phagosomal proteins have been screened for possible posttranscriptional modifications, in particular – phosphorylation.

By using subcellular proteomic tools we have identified proteins belonging to a variety of functional classes (Fig. 5-11). A large number of identified proteins are involved in metabolism, protein biosynthesis and catabolism, cytoskeleton and signal transduction. Mitochondrial, stress response, ER and intracellular transport proteins have also been detected. A smaller component of the phagosomal fraction is formed by iron transport proteins and proteins involved in transcription regulation and splicing. Approximately 10 % of all identified proteins have no known or predicted function.

Most of the identified polypeptides are identical with the recently described latex-beads *D. discoideum* phagosomal proteins. However, a large class of small GTPases, dynamin, involved in vesicle fusion, and heterotrimeric G protein have been not found in *Legionella* associated phagosomal fraction. This difference may be due the used isolation procedures or reflect specific processes induced by *Legionella*. Ad notam, the set of Rab small GTPases and LAMP proteins family were detected in the proteome of human neutrophils after applying an additional approach for identification membrane proteins following phase partition in Triton X-114. The utilization of

the latest generation of detergents as well as various extraction methods were also useful to obtain a more comprehensive list of phagosomal proteins (Garin et al. 2001; Gotthardt et al. 2006a; Gotthardt et al. 2002). Because of the difficulty involved with this technique, it will be necessary in the future to expand the study by identifying membrane proteins and particularly transmembrane proteins by using additional isolation steps.

The detailed characterisation and possible role of the identified phagosomal proteins in *Legionella* will be described below.

6.4.1 Cytoskeleton organisation proteins

We have found a number of proteins involved in cytoskeletal organisation. Comparative analysis of *Legionella*-containing and latex beads-containing phagosomes revealed that most of the molecules involved in actin polymerisation are present only in bacteria-containing phagosomes (Table 6-1) (Gotthardt et al. 2006a). There are components of Arp2/3 complex, actin-like and actin binding proteins.

Table 6-1 Distribution of cytoskeleton organisation and signal transduction proteins in *Legionella*- and latex beads-containing *D. discoideum* phagosomes

Name of protein	Acc N.	Description	*Legionella* phagosome	Latex-beads phagosome
AbpB	**DDB0214810**	actin bundling protein	+	
AclA	**DDB0219936**	actin-like protein	+	
AcpA	**DDB0191202**	actin capping protein	+	
AcpB	**DDB0191243**	actin capping protein	+	
Act	**---**	Actin	+	+
ArpH	**DDB0191138**	Arp2/Arp3 complex	+	
CadA	**DDB0191175**	calcium-dependent cell adhesion	+	+
CdcD	**DDB0191154**	Cell division cycle protein 48	+	
CoaA	**DDB0215369**	Coactosin	+	+
CofA	**DDB0214987**	Cofilin	+	
CorA	**DDB0191115**	Coronin	+	+
CrtA	**DDB0191384**	calreticulin, Ca2+-binding protein	+	+
CtxB	**DDB0185031**	CORTEXILLIN II	+	
FttB	**DDB0190707**	protein kinase C inhibitor activity	+	
GpbA	**DDB0185046**	G protein b-subunit	+	+
HatA	**DDB0215335**	Hisactophilin	+	+
HatB	**DDB0215336**	Hisactophilin II	+	+
JC2V2_0_0324	**DDB0203397**	actin bundling protein	+	
NxnA	**DDB0191502**	annexin VII	+	+
PgkA	**DDB0191349**	phosphoglycerate kinase, calmodulin-binding	+	+
PkiA	**DDB0216234**	similar to protein kinase C inhibitor	+	+
RanA	**DDB0215409**	GTP-binding nuclear protein Ran	+	
RdiA	**DDB0216235**	Rho GDP-dissociation inhibitor	+	
SevA	**DDB0188380**	Ca2+-dependent F-actin fragmenting protein	+	+
SmlA	**DDB0191525**	SMaLl aggregates	+	
TubA	**DDB0191380**	alpha tubulin	+	+
TubB	**DDB0191169**	beta tubulin	+	

Calreticulin, coronin, coasticin and hisactophilin were detected in both *Legionella* and latex beads-

containing phagosomes. Therefore it can be assumed that these molecules are recruited to the phagocytic cup during phagocytosis.

Legionella exploits the cytoskeleton to invade the host, as well as for intracellular movement and modification of phagosome. By using drugs such as Cytochalasin A and latrunculin that interfere with the F-actin formation it was demonstrated that *D. discoideum* utilises conventional phagocytosis to internalise *Legionella*. *D. discoideum* mutants lacking certain actin binding proteins are deficient in bacterial multiplication (Fajardo et al. 2004). Moreover, the confocal microscopy studies on selected host proteins, labelled with GFP, confirmed that the entry of *L. pneumophila* is an actin-mediated process (Lu and Clarke 2005).

The host defence mechanisms against different pathogens, such as *Listeria*, *Rickettsia*, *Shigella*, as well as vaccinia virus include actin cytoskeleton rearrangements (Goldberg 2001). Reflecting this, many of the proteins found in the phagosomal fraction clearly play a role in regulation of phagocytosis by reorganization of the actin cytoskeleton. It may be suggested that pathogens may somehow exploit different cell regulatory pathways that normally controls the nucleation of actin filaments (Fig. 6-1).

Understanding the role of the actin cytoskeleton in phagocytosis and bacterial pathogenicity made a giant leap forward with the discovery of the Arp2/3 complex. Arp2/3 plays a major role in the regulation of actin nucleation. This complex consists of seven subunits, two of which are Actin-related proteins (Arp2 and Arp3), and five of which are unique and initiate growth of a new filament at a distinctive 70 degree angle from the mother filaments. These “branches” are important for different sets of cellular processes like locomotion, intracellular motility of lipid vesicles and phagocytosis. Capping proteins limit actin polymerization to the region activated by the Arp2/3 complex and the elongated filament ends are recapped to prevent depolymerisation and thus conserve the actin filament (Goldberg 2001). Observed in phagosome, actin-associated cofilin was described to have both actin-severing and -depolymerising activities. This small phosphoinositide sensitive protein brings two filaments of actin together by joining actin subunits. Under certain conditions it fragments the filaments and accelerates actin subunit dissociation from their ‘pointed’ (minus) ends.

Normally, actin nucleation and polymerization is initiated via the small guanosine triphosphatase (GTPases) Rac, WAVE and Arp2/3 complex. The process involves several other proteins: (1) cofilin; (2) capping protein, which caps the barbed ends of actin filaments; (3) profilin, providing actin monomers to growing barbed ends; (4) α-actinin, which cross-links actin filaments; and (5) VASP, (vasodilator-stimulated phosphoprotein), which may act as an anticapping protein at the barbed ends. Most of the described actin nucleation machinery components were found in *Legionella* phagosome fraction.

Another actin binding phagosomal component, coronin, was shown to be localised in the

Legionella nascent phagocytic cap, but dissociates immediately after internalization (Lu and Clarke 2005). This protein directly inhibits Arp2/3 complex activity and actin nucleation in the absence of preformed actin filaments (Humphries et al. 2002). Interestingly, coronin-null mutants show defects in phagocytosis and pinocytosis in suspension. On the other hand, the growth of wild-type *L. pneumophila* as well as *Mycobacterium marinum* within in the coronin deficient *D. discoideum* mutant is 10-fold-higher than in wild-type (Solomon et al. 2003; Solomon et al. 2000). Moreover, the interaction of coronin with oxidase specific proteins, p47phox (phagocyte oxidase) and p67phox, important for activation of superoxide production was previously shown. The Phox proteins and coronin have a similar distribution in the cell and both accumulate around the phagocytic vacuole (Grogan et al. 1997). Most likely the cytoskeleton machinery and production of the superoxide during pathogenic infection are co-regulated. This could explain why the phagocytosis rate is low (coronin regulates the cytoskeleton, involved in particle uptake and general phagocytosis event) and multiplication rate is high (absence of connection between superoxide generation machinery and phagocytic cap/cytoskeleton). The role of oxidoreductase machinery, microbicidal function of phagosomes and initiation of immune defence against infections will be discussed below.

An additional candidate for the cytoskeleton reorganization is the actin-binding protein hisactophilin, which is present in two isoforms in *D. discoideum*. Both isoforms are independently transcribed, carry introns at the same position and can be subjected to posttranscriptional modification-myristoylation. This protein could enter via myristic acid component into lipid layers and connect the actin cortex with the plasma membrane. Experimentally, it was found that the binding can be controlled by very small changes in pH (Lund et al. 2005). It is most likely because of the high histidine content that this protein binds to actin in a pH-sensitive manner (Houliston et al. 2002; Stoeckelhuber et al. 1996). The four separated spots, identified as hisactophilin on our master phagosomal gel, could reflect this modification (Fig 5-12). The fact that this protein is membrane-associated suggests that the function of the hisactophilin is to serve as a link between the cortical actin cytoskeleton and the membrane.

Furthermore, annexin VII (ANXA7; ANX7; synexin) was detected. Annexin belongs to the family of phospholipid binding proteins implicated in calcium dependent membrane fusion events. Pittis & Garcia have proposed that annexin plays a role in the phagolysosomal maturation of *Mycobacteria* - containing phagosomes (Pittis et al. 2003). By using confocal and electron microscopy it was found that annexin mainly localized as patches in the cytoplasm of uninfected cells. However, upon phagocytosis of yeast or *E. coli* annexin rapidly translocates and concentrates around the respective phagosomes. On the other hand, annexin was never detected around live *B. suis* pathogen-containing phagosomes. Based on this observation it was suggested, that this protein could be involved in phagosome maturation which might be impaired by some intracellular pathogens (Harricane et al. 1996).

Probably *Legionella* uses the host annexin for its advantage. We speculate that the phagosomal annexin binds phosphoinositol and accumulates it in the *Legionella* phagosomal membrane, thereby inhibiting the fusion with lysosomes. Before this event, *Legionella* may manipulate Rho GTPase in order to anchor the PIP (Fig. 6-1).

A set of proteins including coastosin, severin and actin caping and binding proteins have been shown to be directly or indirectly involved in actin rearrangement and phagocytosis.

It was demonstrated that the nascent phagosome is rapidly transported about the cell on microtubules (Lu and Clarke 2005). According to this observation, residual amounts of both α and β subunits of the tubulin were found, which are probably isolated together with the associated phagosomes. It could be suggested that the tubulin movement may not just promote the transportation of the phagosome from the periphery of the cell, as it was described before, but also facilitate the fusion of *Legionella* phagosome with other endocytic vesicles.

Previously it was shown that two ER residence and cytoskeleton-associated proteins, calnexin and calreticulin, modulate uptake and growth of *L. pneumophila* in *D. discoideum* (Fajardo et al. 2004). Both proteins have been characterized as calcium storage proteins and several studies have indicated that changes in the concentration of calcium affects ER functions. Despite the fact that we have detected calnexin in the phagosomal fraction by specific antibodies, it was not found in the 2D gel. It is very likely that the calnexin, displaying a transmembrane domain, was lost during preparation of the protein fraction or during 2D gel electrophoresis. Conversely, calreticulin is abundant in the phagosomal fraction. This protein is an ER luminal protein with a K(H)DEL recognition signal. It is shown to bind misfolded proteins and prevent them from being exported from the ER to Golgi apparatus.

6.4.2 Signal transduction proteins

The most intriguing molecules in the phagosomes are the proteins which take part in cell signalling pathways. They are likely targets for bacterial secreted effectors. In *Legionella* phagosomes we have found GTP-binding protein Ran (RanA), β-subunits of G protein (GpbA), Rho GDP-dissociation inhibitor (RdiA), two proteins with protein kinase C inhibitor activity (FttB and PkiA) and calmoduline-binding phosphoglycerate kinase (PgkA).

So far it is known that *Legionella* is able to manipulate the host phosphoinositol metabolism to subvert vesicle traffic in eukaryotic host cells by producing a protein called RalF. This effector molecule functions as an exchange factor for the ADP ribosylation factor (ARF) family of GTPases (Nagai et al. 2002; Weber et al. 2006). Generally, during the first steps of phagocytosis, particle binding triggers the local recruitment and activation of one or several Rho proteins, which mediate the nucleation and the dendritic assembly of actin filaments and enrichment of this protein about the nascent phagosome in an Arp2/3-dependent manner (Pollard and Borisy 2003). For optimal

phagocytosis the delivery of endosome-derived membranes to forming phagosomes, controlled by an Arf-family member, Arf6 and by Rab proteins, is required. The GTPase-controlled pathways leading to membrane delivery and actin polymerisation are independently activated upon particle binding. The Rab7 GTPase in *D. discoideum* has been shown to regulate delivery of lysosomal enzymes from endosomal compartment. However this protein is not implicated in delivery of the proton pump proteins, regulating phagosomal pH (Buczynski et al. 1997). The small GTP-binding proteins play a crucial role not only in phagocytic uptake but also in several responses associated with phagocytosis, such as activation of the NADPH oxidase and phagosome maturation (Fig 6-1)(Abo et al. 1991; Knaus et al. 1991).

In addition, kinases are known to be involved in many signalling pathways and have a potentially regulatory function in the endocytic pathways. Despite the calmodulin binding phosphoglycerate kinase, which most likely participates in glycolysis, several kinase inhibitors have been found. Two of them, FttB and PkiA, display the protein kinase C inhibitor activity. Protein kinases C are Ca^{2+} - and phospholipid-dependent protein kinases (PKCs). They represent a family of second

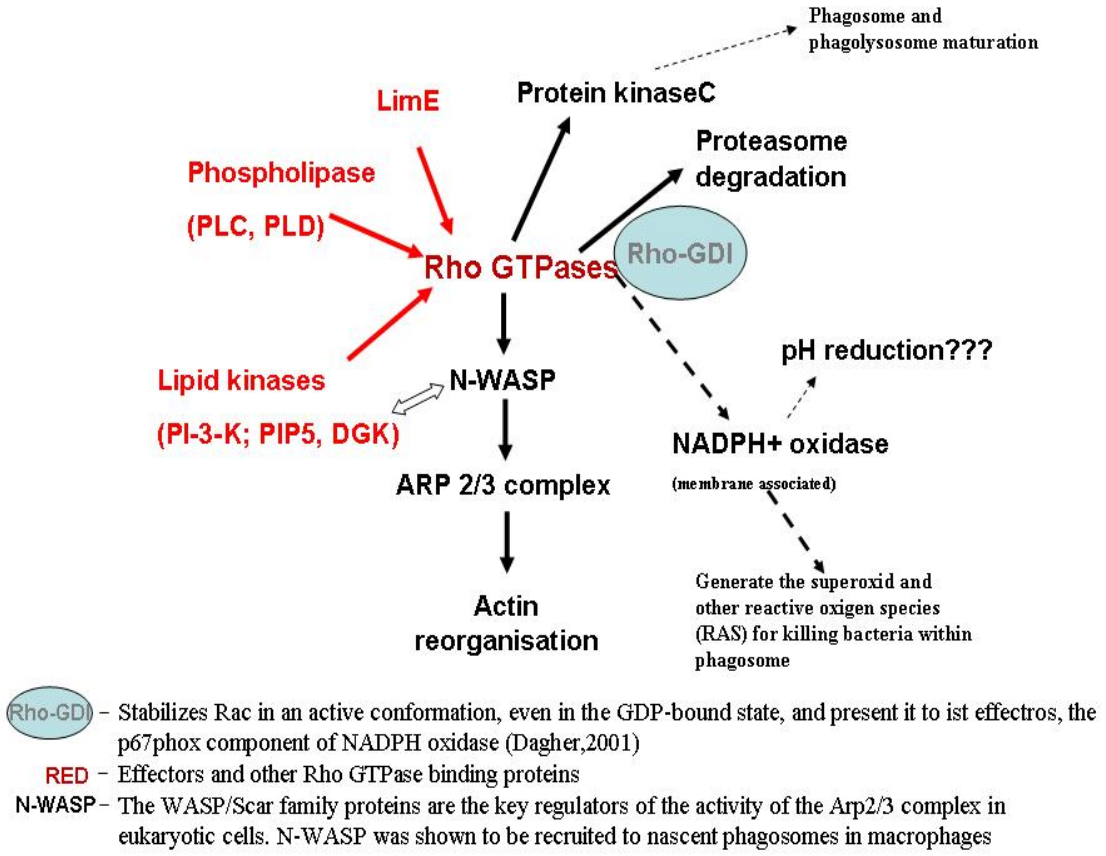


Fig. 6-1 Predicted scheme of host protein interaction during the phagocytic event

messenger-dependent protein kinases that are stimulated by Ca^{2+} and/or phospholipid (Newton 1995). PKS is not a single enzyme but a family of enzymes transducing a number of signals that

promote lipid hydrolysis. The prevalence of this enzyme family in signaling is exemplified by the diverse transduction mechanisms that result in the generation of protein kinase C activator, diacylglycerol. An interesting component, present in large amounts in the *L. hackeliae* phagosome is the inhibitor of Rho GTPases (RDI). This protein has been implicated in cytokinesis through the regulation of the Rho family GTPases Rac1s and (or) RacE in *D. discoideum* cells. Despite this it was reported that in yeast *Saccharomyces cerevisiae* it regulates the activation-inactivation cycle of Rho small GTPases, such as Cdc42 and RhoA, by extracting them from the membrane (Forget et al. 2002; Imai et al. 2002). Moreover, inhibition of Rho A by expression of either the *Clostridium botulinum* C3 transferase or the dominant negative RhoA resulted in enhanced ER transcriptional activation, thus phenocopying the effect of RDI expression on ER transactivation.

Activated Rho proteins are able to initiate signaling pathways controlling reorganization of the actin cytoskeleton (Hall 1998). They also interact with downstream effectors that regulate diverse cellular functions, including oxidant generation, apoptosis, membrane trafficking, cell cycle control and gene expression. RDI interacting with Rho proteins inhibits the nucleotide dissociation and therefore plays a crucial role in the shuttling of Rho GTPases between the cytoplasm and membranes (Fig 6-1).

6.4.3 Proteins involved in biosynthesis and catabolism

The molecules involved in protein biosynthesis and catabolism make up the bulk of phagosomal proteome. The presence of elongation factors, ribosomal proteins, some of tRNA-synthetases as well as proteins typically associated with ER is consistent with the fact that *Legionella* resides in a compartment derived from rough ER. The transcriptional analysis of *Legionella* infected *D. discoideum* cells also displays the upregulation of transcripts, involved in protein biosynthesis (Farbrother et al. 2006).

Surprisingly, several components of the biosynthesis machinery are actin-binding proteins. For example, the elongation factors eEF1α (ABP50 -actin binding protein 50) and eEF1β were found to increase the amount in the cytoskeletal fraction during osmotic stress (Zischka et al. 1999). Moreover, it was shown that *Legionella* produces a glucosyltransferase, which modulates the mammalian elongation factor eEF1α and, as a result, inhibits the protein biosynthesis and causes death of target cells (Belyi et al. 2006).

6.4.4 Vacuolar H^+-ATPases and oxidoreductase activity proteins

The vacuolar H^+-ATPases are highly conserved multisubunit enzymes, which are a part of the molecular machinery that acidifies subcellular compartments and have an important role in various membrane-transport processes. The V-ATPases also participate in receptor-mediated endocytosis, intracellular targeting of lysosomal enzymes, protein processing and degradation, as well as

transport of small molecules across the plasma membrane of various cell types. Recently it was found that pre-existing endosomes that contained the V-ATPase in their membrane, cluster and then fuse with a new phagosome membrane, delivering their contents to the lumen and the V-ATPase to the membrane of the phagosome (Clarke and Maddera 2006a). The integral V_0 domain has been proposed to have a direct role in membrane fusion (Arata et al. 2002; Nishi and Forgac 2002; Sultana et al. 2005).

In contradiction to the study of Lu & Clarke, which claims that the vacuolar H^+-ATPases does not associate with the mature *Legionella* replication vacuoles (Lu and Clarke 2005) we have found a small amount of vacuolar H^+-ATPase's A and B subunits. On the other hand detection of VatA (one of subunit of V-ATPase) was not successful in the phagosomal fractions with specific antibodies (data not shown). At the same time latex-beads *D. discoideum* phagosomal proteome map contains numerous spots reflecting different subunits of V-ATPase (Vat A, B, M and E) (Gotthardt et al. 2006a). This finding could have several explanations. First of all, it may reflect the fact that only residual amounts of this mutlisubunit complex were present and *Legionella* somehow eliminate the machinery, which cause acidification of their compartment. Secondly, the V-ATPase complex could recycle in the phagosomal membrane and therefore this complex is not detectable at all analysed time points.

Nevertheless, the V-ATPase could be present in inactivated form in phagosomal membrane. The pathogen might alkalinize the phagosome to counteract the effects of the V-ATPase or might inhibit the accumulation of functional V-ATPase in the phagosomal membrane either by inactivating the V-ATPase present in the phagosomal membrane (by direct interactions or by enhancing degradation of the enzyme) or by inhibiting fusion with vesicles containing the V-ATPase (Strasser et al. 1999).

Several intracellular pathogens use stratagies to survive and multiply within the phagosomes similar to *Legionella*. *Histoplasma capsulatum*, *Mycobacterium spp*., *Yersinia pseudotuberculosis* are the pathogens that inhibit the phagosomal acidification (pH within phagosomes remained at about 6.0-6.5) and block phagolysosomal maturation (Strasser et al. 1999).

For example *Y. pseudotuberculosis*, which survives and multiplies in the phagosomes of mouse macrophages, was shown to block the phagosomal acidification through the inhibition of vacuolar H^+-ATPase activity. Despite the fact that the amount of A and B subunits of phagosomal V-ATPase was not significantly different between live and dead bacteria infection, the phagosomes containing live bacteria had a 10-fold smaller V-ATPase activity than those containing the dead bacteria (Tsukano et al. 1999)

The proteins with the oxidoreductase activity are characterised by Gene Ontology (GO) annotation as enzymes that catalyse an oxidation-reduction (redox) reaction, a reversible chemical reaction in which the oxidation state of an atom or atoms within a molecule is altered. One substrate acts as a

hydrogen or electron donor and becomes oxidized, while the other acts as hydrogen or electron acceptor and becomes reduced (molecular function ontology).

The superoxide-production is normally activated during phagocytosis to destroy ingested microbes. Interestingly, superoxide dismutase (SodA) and catalase (CatA) are presented in *Legionella* phagosomes. The function of these proteins is neutralization of oxidoreductase activity by removal of superoxide radicals (see reaction in Fig. 8-2 or http://dictybase.org/pathways/DICTY/NEW-IMAGE?type=PATHWAY&object= DETOX1-PWY&detail-level=3). The components, directly involved in oxidoreductase activity, the adaptor protein P40phox and the essential oxidase activator p67phox, were not found in the phagosomal fraction. It was suggested that proteins are sorted to different intracellular compartments via pleckstrin homology (PH) and phagocyte oxidase (phox) homology (PX) domains. These domains are recognized by specific phosphoinositides, recruiting proteins to appropriate cell membranes. Interactions between phosphoinositides and proteins are regulated by kinases or phosphatases (Lemmon 2003).

From EM study measuring the thickness of phagosomal and ER membranes of *Legionella* infected cells it was concluded that phagosomes are altered in their lipid composition. This alteration occurs rapidly between 5 and 15 minutes after infection (Tilney et al. 2001). It is now well established that the plasma membrane, as well as the endosomal, the lysosomal and trans-Golgi membranes, contain cholesterol and sphingolipids. Neither of these components was present to any extent in membranes of ER.

6.4.5 Other classes of proteins

A modest amount of the phagosomal fraction consists of mitochondrial, proteasome and folding proteins. The identification of 9 mitochondrial proteins could have two different explanations. First, the morphological description revealed a physical attachment of ER vesicles and mitochondria to phagosome-enclosed bacteria, followed by association with the ribosome (Tilney et al. 2001). Second, all mitochondrial proteins founded in the phagosome are present in minor amount, which may indicate a possible contamination of the phagosomal fraction by mitochondria. Evidence could come from imaging of GFP-fused mitochondrial molecules in *Legionella* infected *D. discoideum* cells.

Some of the proteins identified in *Legionella* phagosome were components of proteasome subunits. Normally, proteins wich fail to fold properly as well as constitutive or regulated short-lived proteins of the ER are subjected to proteolysis by cytosolic 26S proteasome. Polyubiquitination modifies the target protein for its subsequent degradation by the 26S proteasome. A recent publication that described the human neutrophil phagosome proposes that protein processing and quality control machinery functions within the phagosomes to modify it for antigen presentation (Burlak et al. 2006). Taking together, the proteome data and recently published transcriptional data

clearly show activation of genes encoding ubiquitin and other proteasome components, as well as genes encoding for components of the biosynthesis machinery. This suggests that *Legionella* may exploit the metabolism of its host in order to obtain a source of amino acids (Farbrother, 2006).

Interestingly, annotation of the *Legionella* genomes reveals several eukaryotic-like proteins with F-box or U-box domains (Bruggemann et al. 2006). Domains of the first class were characterized as components of SCF ubiquitin-ligase complexes (named after their main components, Skp I, Cullin, and F-box protein), in which they bind substrates for ubiquitin-mediated proteolysis. The second class consists of ubiquitin-protein ligases. Using yeast two-hybrid technology, all mammalian U-box proteins have been reported to interact with molecular chaperones or co-chaperones. This confirmed that the function of U-box is to mediate degradation of unfolded or misfolded proteins (Hatakeyama et al. 2004). Recent reports suggest that exploitation of the host proteasome machinery could be a general mechanism used by bacteria to program the destruction of a T3/4SS effector when its function in the host cell is no longer required by these eukaryotic-like proteins (Angot et al. 2007).

Almost 10 % of the identified phagosomal proteins (15) are hypothetical proteins. Elucidation of their role, molecular characterisation and annotation of predicted interaction of these proteins with the known components of phagosome would be interesting for better understanding of phagosome functions.

Taking together, the *Legionella* phagosomes accumulate proteins from different cellular compartments with a wide range of molecular and cellular functions. Identification of the components of ER, mitochondria, proteasome, certain components of cytoskeleton reorganisation and protein bisynthesis machinery, as well as stress response and metabolism proteins shows that phagosomes are closely associated with other intracellular components. The detailed model of the *Legionella*-phagosome and complex interaction between phagosomal proteins is represented in Fig.6-2.

Most of the phagosomal components are common for latex beads and *Legionella*-containing phagosome. However, the regulatory components, like RanA, FttB and RdiA appear only in bacteria-containing phagosomes. The proteins involved in removal of superoxide radicals during response to oxidative stress are also present only in the *Legionella*-containing compartment. Additionally, the identification of larger amounts of actin cytoskeleton and regulatory components in bacterial phagosome, versus in latex beads-containing phagosomes probably indicates that *Legionella* utilize actin network to its advance. This could include movement of bacterial phagosome along actin filaments and (or) permission to fuse with other endocytic components.

6.5 Alterations of the phagosomal composition

6.5.1 Species-specific phagosomal proteome variation

Since it has been previously shown that two employed *Legionella* strains have a distinct behaviour within the host cell, the variation in the phagosomes protein profiles was examined. Modulations of the phagosomal proteome of *L. pneumophila* Corby versus *L. hackeliae* were shown in Table 5-3. The most prominent proteins differences will be discussed in the following section.

First of all, an increase of the cysteine protease inhibitor was observed at early stage of infection in the pathogenic *Legionella* phagosome. Souza et al. have demonstrated that vesicle-associated cysteine proteases play a major role in digestion of bacteria in vacuoles (Souza 1997). *D. discoideum* cysteine proteases as well as V-ATPase were delivered to nascent latex beads-containing phagosomes within 5 min of ingestion and remained enriched during long periods of chase (Rezabek, 1997, Rupper 2001). In our study the presence of a residual amount of some V-ATPase subunits was observed, but proteases or lysosomal enzymes, modifying phagosomal pH were not detected.

Secondly, the members of protein kinase C superfamily were proposed to be recruited to the phagosome with variable kinetics and to acquire the microbicidal properties of phagosomes by regulating phagolysosomal fusion. Although this protein kinase is not present in the phagosomal composition, the comparison of *L. pneumophila* Corby and *L. hackeliae* phagosomal proteome reveal two candidates for manipulation of protein kinase C activity. One of them, encoded by *pkiA* appears in larger amount in *L. pneumophila* Corby phagosome at two and four hours after infection, and has a high similarity with protein kinase C inhibitor. Another protein, encoded by *fttB*, is up-regulated in the *L. hackeliae*-containing phagosome. Gene ontology annotation in the *D. discoideum* database reveals several functions of FttB, including diacylglycerol-activated phospholipid-dependent protein kinase C inhibitor activity. This protein seems to be involved in many biological processes, such as DNA damage checkpoint, response to external stimuli, microtubule cytoskeleton polarization and Ras protein signal transduction. It may be proposed that these molecules indirectly regulate the phagosome maturation by manipulation of protein kinase C activity.

The third global change in protein composition concerns signal transduction as well. The disappearance of the Rho GDP-dissociation inhibitor (Rho GDI1) encoded by *rdiA* in the pathogen-containing phagosome was observed. This protein modulates the cycling of Rho GTPases between active GTP-binding and inactive GDP-binding conformation, that prevents interaction with their effectors (Hart et al. 1992). GDIs participate in a number of interactions resulting in formation of a multiprotein complex. A lipid kinase complex, a member of ezrin/radixin/moesin family, components of NADPH oxidase complex and multidomain protein Vav are among the proteins that

Table 6-1 Selected host factor implicated in *Legionella* infection and their alteration during infection (+) presence of protein; (-) absence of protein; (+↑) increase of protein amount in *L. pneumophila* Corby or *L. hackeliae* phagosome; (+↓) decrease of protein amount in *L. pneumophila* Corby or *L. hackeliae*-containing phagosome.

Host factors relevant in *Legionella* infection	*Legionella* phagosome						Latex beads phagosome (Gotthardt et al. 2006a)
	L. pneumophila Corby			*L. hackeliae*			
	2h	4h	6h	2h	4h	6h	
ER residue proteins:							
Calreticulin	+	+	+	+	+	+	+
Calnexin*	-	-	-	-	-	-	-
DisA (G. Roy, 2006)	-	-	-	-	-	-	-
Rab GTPases:							
Rab 1 (G. Roy,2004) Arf1 (G. Roy, 2002) ArfA in *D. discoideum* Sec 22 (G. Roy,2004)	Absent, probably because of isolation procedure						+ +
V-ATPase:							
VatA	+	+	+↑	+	+	+↓	+
VatB	+	+↓	+↑	+	+↑	+↓	+
VatD	-	-	-	-	-	-	+
VatE	-	-	-	-	-	-	+
VatM	-	-	-	-	-	-	+
Cytoskeleton associated proteins:							
Actin	+	+	+	+	+	+	+
Annexin	+	+↑	+	+	+↓	+	+
Arp 2/3 compex components	+	+	+	+	+↑	+	+
Actin binding proteins	+	+	+	+	+	+	-
Actin capping proteins	+	+	+↑	+	+	+↓	-
Coronin	+↑	+	+↑	+	+	+↓	+
Coastosin	+	+	+↑	+	+	+↓	+
Cortexilin	+	+	+↑	+	+	+↓	-
Cofilin	+↑	+↑	+↑	+↓	+↓	+↓	-
Hisactophilin	+	+	+	+	+	+	+
Profiling	-	-	-	-	-	-	-
Tubulin	+	+↑	+↓	+	+↓	+↑	+
Another interesting proteins:							
Discoidin	+	+	+↑	+	+	+↓	+
RtoA**	-	-	-	-	-	-	-
Nramp	-	-	-	-	-	-	-
SodA (superoxide dismutase)	+↑	+↑	+	+↓	+↓	+	-
CxeA (cytochrome c oxidase subunit)	+	+↑	+	+	+↓	+	-
FttB (protein kinase C inhibitor activity)	+↓	+↓	+	+↑	+↑	+	-
RdiA (Rho dissociation. Inhibitor)	+↑↓	+	+	+↑↓	+↑	+↑	-
CpiA (cysteine protease inhibitor)	+↑	+	+	+↓	+	+	-

*** Calnexin was detectible by western blot analysis or by GFP mutant**

**** RtoA protein contains several repeats of a serine-rich motif, which catalyzes the fusion of phospholipid vesicles *in vitro*. Transcription regulated by *Legionella* infection (Farbrother, 2006). Probably not phagosomal associated.**

have been reported to interact with GDI. The *D. discoideum* mutant, lacking RDI1 was not deficient in phagocytosis (Francisco Rivero, 2002). Taking together, it can be assumed that GDI is not involved in the uptake of bacteria, but plays a critical role in the regulation of signal transduction cascades mediated by a subset of Rho GTPases during *Legionella* infection.

6.5.2 Actin degradation caused by *Legionella* infection

Actin is a major component of eukaryotic cells. Despite a number of key functions, this protein is also known as a target for several bacterial toxins. We have found that pathogenic *Legionella* infection causes degradation of actin in the phagosomal fraction. A similar phenomenon was observed in *Salmonella enterica*-infected *Acanthamoeba rhysodes*. The degradation occurred via a virulence-associated SpvB protein, which functions as a mono (ADP-ribosyl) transferase (Tezcan-Merdol et al. 2005).

Numerous bacterial effectors were described to modulate the actin rearrangement. This modulation could happen directly by modification of actin or by targeting the components that are involved in controlling the actin cytoskeleton. The mechanisms of toxin functions, which bind the regulators of actin polymerisation and reorganisation, are well described. Most of them are mediated by Rho GTPase, involved in regulation of actin polymerisation. The first group of toxins can irreversibly inhibit Rho GTPase by ribosylation or glycosylation. For example, *Pasteurella multocida* toxin (PMT) was demonstrated to activate the RhoGTPases in dendritic cells (DC) and to induce changes in DC morphology and actin polymerization (Blocker et al. 2006). Another mechanism of Rho GTPase manipulation uses *Yersinia enterocolitica*. Yop T, secreted by this bacteria causes proteolytic cleavage of Rho GTPase (Aktories and Barbieri 2005). The second group modulates the actin polymerisation via Arp2/3 complexes. Finally, the group of actin binding bacterial effectors is present in a wide range of bacteria. There are *Clostridium botulinum* C2 toxin, *Clostridium perfringens* Iota toxin, and *Clostridium spiroforme* toxin, which cause ribosylation of actin with subsequent depolymerisation of actin filaments and destruction of the microfilament network (Aktories and Wegner 1992). *Vibrio cholerae* RTX toxin causes depolymerization of actin stress fibers and covalent cross-linking of cellular actin into dimers, trimers and higher multimers (Fullner and Mekalanos 2000). *Salmonella typhimurium* is known to produce the SipA effector that functions as an inhibitor of cytoskeletal reorganization via both ADF/cofilin- and gelsolin-directed actin disassembly. SipA binds host actin, enhances its polymerization near adherent extracellular bacteria, and contributes to cytoskeletal rearrangements that internalize the pathogen (Lilic et al. 2003).

As previously mentioned, only one of the known bacterial effectors (SpvB) initiates the actin degradation. As we have observed a similar actin degradation pattern in the case of *L. pneumophila* Corby infection, we can speculate that *Legionella* secrete some effector(s), which also cause(s) this effect.

It is known that phagosome-membrane-assembled actin filaments facilitate fusion with late endocytic organelles (Kjeken et al. 2004). The non-pathogenic *Mycobacterium*-containing phagosomes were shown to nucleate actin in a manner very similar to the latex beads-containing phagosomes. In contrast the pathogenic *Mycobacterium*-containing phagosomes, whose maturation is blocked in macrophages, were unable to assemble actin in vitro. In addition, signalling events that regulate phagosome actin assembly were found to be regulated by different lipids (Anes et al. 2003). More recently, the level of intracellular cAMP was shown to regulate actin assembly and thereby phagosomal fusion events (Kalamidas et al. 2006). Increasing cAMP concentration inhibited assembly of phagosomal actin, preventing the fusion with acidic compartment, and was associated with increased replication of pathogen, and vice versa. Interestingly, the anti *L. pneumophila* activity of macrophages induced by lipopolysaccharide (LPS) was reversed *in vitro* by treatment with dibutyryl cAMP (DcAMP), which increases intracellular levels of cAMP. Furthermore, the same effect was shown by treatment with prostaglandin E2, colchicine, isoproterenol, theophylline, or hydrocortisone, all of which are known to increase the intracellular levels of cyclic AMP in various tissues (Egawa et al. 1992). All these observations suggest that degradation of monomeric actin caused by *Legionella* could prevent the filamentous actin nucleation and therefore fusion of bacterial phagosomes with acidic organelles.

6.5.3 Time-specific phagosomal proteome variation

The maturation of phagosome is characterised by changes in the molecules associated with phagosomal membrane. To determine how far protein expression patterns really are a consequence of pathogen-induced events we have compared maturation of *L. pneumophila* Corby-containing phagosomes with phagosomes containing *L. hackeliae*.

Kinetic analysis clearly showed that the *L. pneumophila* Corby phagosome accumulated ER markers and ribosomal constituents including several elongation factors. At the same time points the degradative *L. hackeliae* phagosome eliminated components of the protein biosynthesis machinery (Table 5-4, 5-5). In both type of phagosomes at six hours post infection the membrane slightly sheds the actin binding protein, hisactophilin. This protein functions as a pH sensor in the plasma membrane by reversibly connecting the membrane with the actin network upon local changes of the proton concentration (Stoeckelhuber, Noegel, 1996).

Many factors, like lipids (including phosphoinositides), small GTPases, signalling and actin dynamics are involved in maturation of *Legionella* phagosomes in macrophages and *D. discoideum* (Lu and Clarke 2005; Weber et al. 2006). Exciting progress has been made by the analysis of latex-bead-containing *Dictyostelium* phagosomes which helped to define three maturation stages (Gotthardt et al., 2002). The first maturation stage is characterized by coronin and lysosomal glycoprotein (LmpB) acquisition. The second stage is characterized by the transfer of lysosomal enzymes. The third stage is characterized by quantitative retrieval of hydrolases from the

phagolysosome and exocytosis of latex beads. A subsequent proteomic study has been enlightening since almost 200 phagosomal proteins were identified and ordered by their temporal appearance (Gotthardt et al., 2006). Nevertheless, the latex beads phagosome dynamics analyses can not be completely applied to *Legionella*-containg phagosome. There is evidence in the literature that maturation process of bacterial pathogens is different from „normal" phagocytosis. In case of *Legionella* the acquisition of lysosomal proteins and phagosomal acidification are delayed and correlated with the bacterial-replication phase.

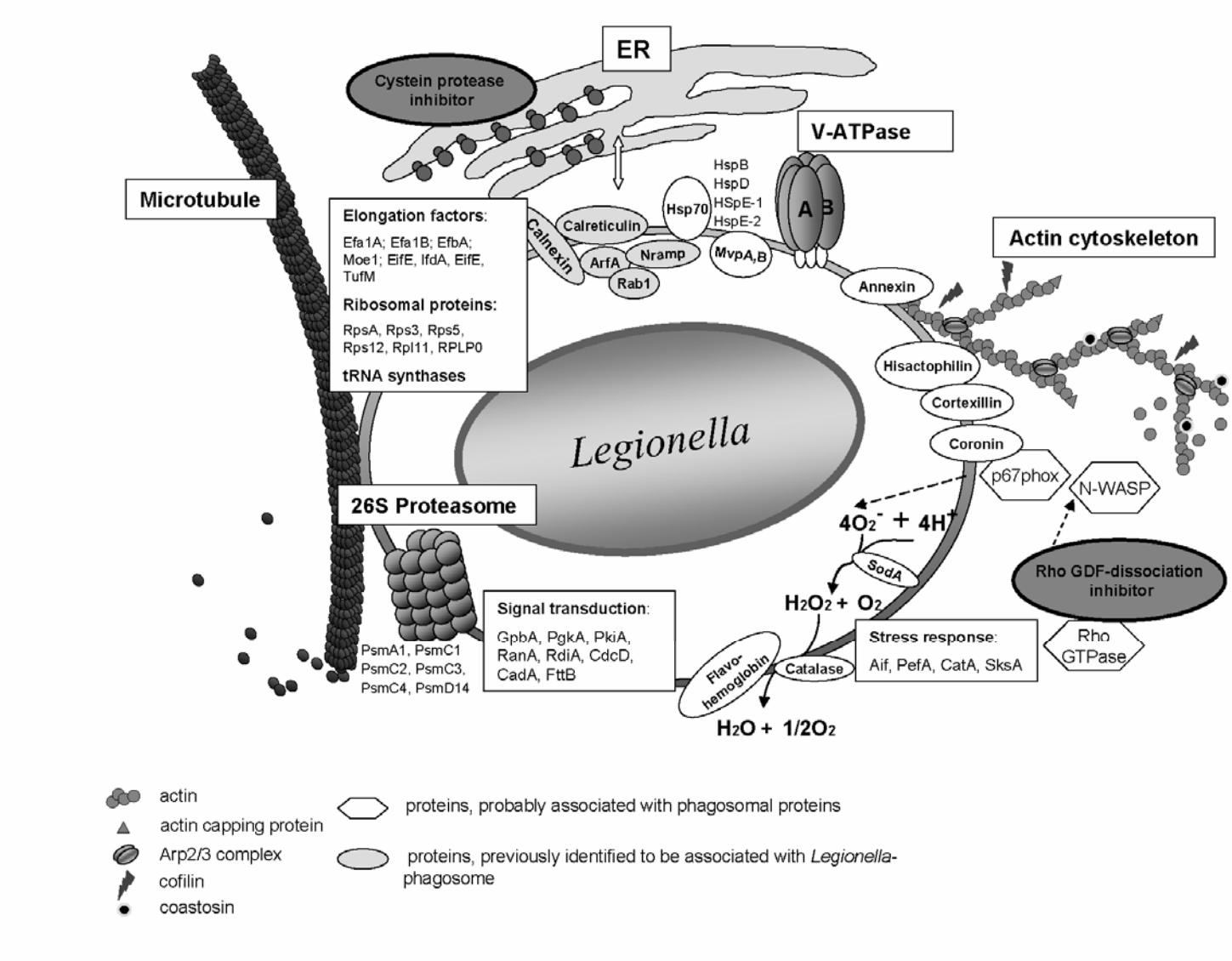


Fig. 6-2 Predicted composition of *Legionella*-containing *D. discoideum* phagosome. The selected proteins separated into classes lead to molecular mechanisms governing phagosome functions. For some identified proteins their potential interactions with other cell components are depicted. The proteins not indicated in the figure are 15 unknown proteins, 31 metabolism proteins and 10 mitochondrial proteins. Whole phagosomal proteins are listed in Table 3-2

6.6 Conclusion

The interactions between host and pathogen during *Legionella* infection are very complex. Therefore, the study of molecular mechanisms of intracellular survival and replication of the pathogen are still in progress.

In the present work two new screening methods were established for analysis of *Legionella* virulence and host-pathogen interaction using *D. discoideum* as a model system. Furthermore, to understand the processes taking place during infection, a protocol was developed for isolation of the *Legionella*-containing phagosome from infected *Dictyostelium* cells. The present method resulted in the construction of a phagosomal protein map (the suggested phagosomal model is presented in Fig. 6-2).

Analysis of the phagosomal proteome alteration between *Legionella* species with different intracellular phenotypes and during infection revealed specific differences. This analysis provided a reference flow chart of the *Legionella*-phagosome maturation and shed light on the molecular mechanisms by which the pathogen manipulates its host. In addition to proteins with an evident role in phagosome maturation, proteins were identified for which a link to phagosome activities remains to be established. Nevertheless, the first proteomic analysis of a pathogen-containing phagosome will surely open new avenues for future projects.

7 References

Abo A, Pick E, Hall A, Totty N, Teahan CG, Segal AW. 1991. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. Nature 353(6345):668-70.

Abu Kwaik Y. 1996. The phagosome containing *Legionella pneumophila* within the protozoan *Hartmannella vermiformis* is surrounded by the rough endoplasmic reticulum. Appl Environ Microbiol 62(6):2022-8.

Abu Kwaik Y, Gao LY, Stone BJ, Venkataraman C, Harb OS. 1998. Invasion of protozoa by *Legionella pneumophila* and its role in bacterial ecology and pathogenesis. Appl Environ Microbiol 64(9):3127-33.

Adeleke A, Pruckler J, Benson R, Rowbotham T, Halablab M, Fields B. 1996. *Legionella*-like amebal pathogens--phylogenetic status and possible role in respiratory disease. Emerg Infect Dis 2(3):225-30.

Aktories K, Barbieri JT. 2005. Bacterial cytotoxins: targeting eukaryotic switches. Nat Rev Microbiol 3(5):397-410.

Aktories K, Wegner A. 1992. Mechanisms of the cytopathic action of actin-ADP-ribosylating toxins. Mol Microbiol 6(20):2905-8.

Alli OA, Gao LY, Pedersen LL, Zink S, Radulic M, Doric M, Abu Kwaik Y. 2000. Temporal pore formation-mediated egress from macrophages and alveolar epithelial cells by *Legionella pneumophila*. Infect Immun 68(11):6431-40.

Anes E, Kuhnel MP, Bos E, Moniz-Pereira J, Habermann A, Griffiths G. 2003. Selected lipids activate phagosome actin assembly and maturation resulting in killing of pathogenic mycobacteria. Nat Cell Biol 5(9):793-802.

Angot A, Vergunst A, Genin S, Peeters N. 2007. Exploitation of eukaryotic ubiquitin signaling pathways by effectors translocated by bacterial type III and type IV secretion systems. PLoS Pathog 3(1):e3.

Aragon V, Kurtz S, Flieger A, Neumeister B, Cianciotto NP. 2000. Secreted enzymatic activities of wild-type and pilD-deficient *Legionella pneumophila*. Infect Immun 68(4):1855-63.

Aragon V, Rossier O, Cianciotto NP. 2002. *Legionella pneumophila* genes that encode lipase and phospholipase C activities. Microbiology 148(Pt 7):2223-31.

Arata Y, Nishi T, Kawasaki-Nishi S, Shao E, Wilkens S, Forgac M. 2002. Structure, subunit function and regulation of the coated vesicle and yeast vacuolar (H(+))-ATPases. Biochim Biophys Acta 1555(1-3):71-4.

Bachman MA, Swanson MS. 2004. The LetE protein enhances expression of multiple LetA/LetS-dependent transmission traits by *Legionella pneumophila*. Infect Immun

72(6):3284-93.

Baine WB. 1985. Cytolytic and phospholipase C activity in Legionella species. J Gen Microbiol 131(6):1383-91.

Bandyopadhyay P, Byrne B, Chan Y, Swanson MS, Steinman HM. 2003. *Legionella pneumophila* catalase-peroxidases are required for proper trafficking and growth in primary macrophages. Infect Immun 71(8):4526-35.

Bandyopadhyay P, Xiao H, Coleman HA, Price-Whelan A, Steinman HM. 2004. Icm/dot-independent entry of *Legionella pneumophila* into amoeba and macrophage hosts. Infect Immun 72(8):4541-51.

Barz C, Abahji TN, Trulzsch K, Heesemann J. 2000. The Yersinia Ser/Thr protein kinase YpkA/YopO directly interacts with the small GTPases RhoA and Rac-1. FEBS Lett 482(1-2):139-43.

Baskerville A, Conlan JW, Ashworth LA, Dowsett AB. 1986. Pulmonary damage caused by a protease from *Legionella pneumophila*. Br J Exp Pathol 67(4):527-36.

Becker T, Volchuk A, Rothman JE. 2005. Differential use of endoplasmic reticulum membrane for phagocytosis in J774 macrophages. Proc Natl Acad Sci U S A 102(11):4022-6.

Bellinger-Kawahara C, Horwitz MA. 1990. Complement component C3 fixes selectively to the major outer membrane protein (MOMP) of *Legionella pneumophila* and mediates phagocytosis of liposome-MOMP complexes by human monocytes. J Exp Med 172(4):1201-10.

Belyi Y, Niggeweg R, Opitz B, Vogelsgesang M, Hippenstiel S, Wilm M, Aktories K. 2006. *Legionella pneumophila* glucosyltransferase inhibits host elongation factor 1A. Proc Natl Acad Sci U S A 103(45):16953-8.

Berger KH, Isberg RR. 1993. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. Mol Microbiol 7(1):7-19.

Blocker A, Severin FF, Burkhardt JK, Bingham JB, Yu H, Olivo JC, Schroer TA, Hyman AA, Griffiths G. 1997. Molecular requirements for bi-directional movement of phagosomes along microtubules. J Cell Biol 137(1):113-29.

Blocker D, Berod L, Fluhr JW, Orth J, Idzko M, Aktories K, Norgauer J. 2006. *Pasteurella multocida* toxin (PMT) activates RhoGTPases, induces actin polymerization and inhibits migration of human dendritic cells, but does not influence macropinocytosis. Int Immunol 18(3):459-64.

Brenner DJ, Steigerwalt AG, McDade JE. 1979. Classification of the Legionnaires' disease bacterium: *Legionella pneumophila*, genus novum, species nova, of the family *Legionellaceae*, familia nova. Ann Intern Med 90(4):656-8.

Brieland J, McClain M, Heath L, Chrisp C, Huffnagle G, LeGendre M, Hurley M, Fantone J,

Engleberg C. 1996. Coinoculation with *Hartmannella vermiformis* enhances replicative *Legionella pneumophila* lung infection in a murine model of Legionnaires' disease. Infect Immun 64(7):2449-56.

Bruggemann H, Cazalet C, Buchrieser C. 2006. Adaptation of *Legionella pneumophila* to the host environment: role of protein secretion, effectors and eukaryotic-like proteins. Curr Opin Microbiol 9(1):86-94.

Buczynski G, Bush J, Zhang L, Rodriguez-Paris J, Cardelli J. 1997. Evidence for a recycling role for Rab7 in regulating a late step in endocytosis and in retention of lysosomal enzymes in *Dictyostelium discoideum*. Mol Biol Cell 8(7):1343-60.

Burlak C, Whitney AR, Mead DJ, Hackstadt T, Deleo FR. 2006. Maturation of human neutrophil phagosomes includes incorporation of molecular chaperones and endoplasmic reticulum quality control machinery. Mol Cell Proteomics 5(4):620-34.

Cantalupo G, Alifano P, Roberti V, Bruni CB, Bucci C. 2001. Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes. Embo J 20(4):683-93.

Cazalet C, Rusniok C, Bruggemann H, Zidane N, Magnier A, Ma L, Tichit M, Jarraud S, Bouchier C, Vandenesch F and others. 2004. Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. Nat Genet 36(11):1165-73.

Chen J, de Felipe KS, Clarke M, Lu H, Anderson OR, Segal G, Shuman HA. 2004. *Legionella* effectors that promote nonlytic release from protozoa. Science 303(5662):1358-61.

Chen P, Ostrow BD, Tafuri SR, Chisholm RL. 1994. Targeted disruption of the *Dictyostelium* RMLC gene produces cells defective in cytokinesis and development. J Cell Biol 127(6 Pt 2):1933-44.

Christie PJ, Vogel JP. 2000. Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. Trends Microbiol 8(8):354-60.

Cianciotto NP, Bangsborg JM, Eisenstein BI, Engleberg NC. 1990. Identification of mip-like genes in the genus *Legionella*. Infect Immun 58(9):2912-8.

Cianciotto NP, Eisenstein BI, Mody CH, Toews GB, Engleberg NC. 1989. A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. Infect Immun 57(4):1255-62.

Cianciotto NP, Fields BS. 1992. *Legionella pneumophila* mip gene potentiates intracellular infection of protozoa and human macrophages. Proc Natl Acad Sci U S A 89(11):5188-91.

Cirillo JD, Cirillo SL, Yan L, Bermudez LE, Falkow S, Tompkins LS. 1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. Infect Immun 67(9):4427-34.

Cirillo SL, Yan L, Littman M, Samrakandi MM, Cirillo JD. 2002. Role of the *Legionella pneumophila* rtxA gene in amoebae. Microbiology 148(Pt 6):1667-77.

Clarke M, Maddera L. 2006b. Phagocyte meets prey: uptake, internalization, and killing of bacteria by *Dictyostelium* amoebae. Eur J Cell Biol 85(9-10):1001-10.

Clemens DL, Horwitz MA. 1995. Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. J Exp Med 181(1):257-70.

Conlan JW, Baskerville A, Ashworth LA. 1986. Separation of *Legionella pneumophila* proteases and purification of a protease which produces lesions like those of Legionnaires' disease in guinea pig lung. J Gen Microbiol 132(6):1565-74.

Conlan JW, Williams A, Ashworth LA. 1988. Inactivation of human alpha-1-antitrypsin by a tissue-destructive protease of *Legionella pneumophila*. J Gen Microbiol 134(2):481-7.

Conover GM, Derre I, Vogel JP, Isberg RR. 2003. The *Legionella pneumophila* LidA protein: a translocated substrate of the Dot/Icm system associated with maintenance of bacterial integrity. Mol Microbiol 48(2):305-21.

De Lozanne A, Spudich JA. 1987. Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. Science 236(4805):1086-91.

Debroy S, Aragon V, Kurtz S, Cianciotto NP. 2006. *Legionella pneumophila* Mip, a surface-exposed peptidylproline cis-trans-isomerase, promotes the presence of phospholipase C-like activity in culture supernatants. Infect Immun 74(9):5152-60.

Defacque H, Bos E, Garvalov B, Barret C, Roy C, Mangeat P, Shin HW, Rybin V, Griffiths G. 2002. Phosphoinositides regulate membrane-dependent actin assembly by latex bead phagosomes. Mol Biol Cell 13(4):1190-202.

Dermine JF, Duclos S, Garin J, St-Louis F, Rea S, Parton RG, Desjardins M. 2001. Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. J Biol Chem 276(21):18507-12.

Derre I, Isberg RR. 2004. *Legionella pneumophila* replication vacuole formation involves rapid recruitment of proteins of the early secretory system. Infect Immun 72(5):3048-53.

Derre I, Isberg RR. 2005. LidA, a translocated substrate of the *Legionella pneumophila* type IV secretion system, interferes with the early secretory pathway. Infect Immun 73(7):4370-80.

Desjardins M. 1995. Biogenesis of phagolysosomes: the 'kiss and run' hypothesis. Trends Cell Biol 5(5):183-6.

Desjardins M. 2003. ER-mediated phagocytosis: a new membrane for new functions. Nat Rev Immunol 3(4):280-91.

Desjardins M, Celis JE, van Meer G, Dieplinger H, Jahraus A, Griffiths G, Huber LA. 1994. Molecular characterization of phagosomes. J Biol Chem 269(51):32194-200.

Desjardins M, Houde M, Gagnon E. 2005. Phagocytosis: the convoluted way from nutrition to adaptive immunity. Immunol Rev 207:158-65.

Diederen BM, de Jong CM, Kluytmans JA, van der Zee A, Peeters MF. 2006. Detection and quantification of *Legionella pneumophila* DNA in serum: case reports and review of the literature. J Med Microbiol 55(Pt 5):639-42.

Diederen BM, de Jong CM, Marmouk F, Kluytmans JA, Peeters MF, Van der Zee A. 2007. Evaluation of real-time PCR for the early detection of *Legionella pneumophila* DNA in serum samples. J Med Microbiol 56(Pt 1):94-101.

Dietrich C, Heuner K, Brand BC, Hacker J, Steinert M. 2001. Flagellum of *Legionella pneumophila* positively affects the early phase of infection of eukaryotic host cells. Infect Immun 69(4):2116-22.

Dreyfus LA, Iglewski BH. 1986. Purification and characterization of an extracellular protease of *Legionella pneumophila*. Infect Immun 51(3):736-43.

Duhon D, Cardelli J. 2002. The regulation of phagosome maturation in *Dictyostelium*. J Muscle Res Cell Motil 23(7-8):803-8.

Edelstein PH. 1981. Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. J Clin Microbiol 14(3):298-303.

Egawa K, Klein TW, Yamamoto Y, Newton CA, Friedman H. 1992. Cyclic AMP inhibition of lipopolysaccharide-induced restriction of *Legionella pneumophila* growth in macrophage cultures. Infect Immun 60(5):1936-40.

Eichinger L, Pachebat JA, Glockner G, Rajandream MA, Sucgang R, Berriman M, Song J, Olsen R, Szafranski K, Xu Q and others. 2005. The genome of the social amoeba *Dictyostelium discoideum*. Nature 435(7038):43-57.

Erdos GW, Raper KB, Vogen LK. 1976. Effects of light and temperature on macrocyst formation in paired mating types of *Dictyostelium discoideum*. J Bacteriol 128(1):495-7.

Faix J, Kreppel L, Shaulsky G, Schleicher M, Kimmel AR. 2004. A rapid and efficient method to generate multiple gene disruptions in *Dictyostelium discoideum* using a single selectable marker and the Cre-loxP system. Nucleic Acids Res 32(19):e143.

Fajardo M, Schleicher M, Noegel A, Bozzaro S, Killinger S, Heuner K, Hacker J, Steinert M. 2004. Calnexin, calreticulin and cytoskeleton-associated proteins modulate uptake and growth of *Legionella pneumophila* in *Dictyostelium discoideum*. Microbiology 150(Pt 9):2825-35.

Farbrother P, Wagner C, Na J, Tunggal B, Morio T, Urushihara H, Tanaka Y, Schleicher M, Steinert M, Eichinger L. 2006. *Dictyostelium* transcriptional host cell response upon infection with *Legionella*. Cell Microbiol 8(3):438-56.

Feeley JC, Gibson RJ, Gorman GW, Langford NC, Rasheed JK, Mackel DC, Baine WB. 1979. Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. J Clin Microbiol 10(4):437-41.

Fields BS. 1996. The molecular ecology of *legionellae*. Trends Microbiol 4(7):286-90.

Fischer G, Bang H, Ludwig B, Mann K, Hacker J. 1992. Mip protein of *Legionella pneumophila* exhibits peptidyl-prolyl-cis/trans isomerase (PPlase) activity. Mol Microbiol 6(10):1375-83.

Flieger A, Gongab S, Faigle M, Mayer HA, Kehrer U, Mussotter J, Bartmann P, Neumeister B. 2000. Phospholipase A secreted by *Legionella pneumophila* destroys alveolar surfactant phospholipids. FEMS Microbiol Lett 188(2):129-33.

Flieger A, Rydzewski K, Banerji S, Broich M, Heuner K. 2004. Cloning and characterization of the gene encoding the major cell-associated phospholipase A of *Legionella pneumophila*, plaB, exhibiting hemolytic activity. Infect Immun 72(5):2648-58.

Forget MA, Desrosiers RR, Gingras D, Beliveau R. 2002. Phosphorylation states of Cdc42 and RhoA regulate their interactions with Rho GDP dissociation inhibitor and their extraction from biological membranes. Biochem J 361(Pt 2):243-54.

Franke J, Kessin R. 1977. A defined minimal medium for axenic strains of *Dictyostelium discoideum*. Proc Natl Acad Sci U S A 74(5):2157-61.

Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ, Sharrar RG, Harris J, Mallison GF, Martin SM, McDade JE and others. 1977. Legionnaires' disease: description of an epidemic of pneumonia. N Engl J Med 297(22):1189-97.

Fullner KJ, Mekalanos JJ. 2000. In vivo covalent cross-linking of cellular actin by the *Vibrio cholerae* RTX toxin. Embo J 19(20):5315-23.

Gabay JE, Blake M, Niles WD, Horwitz MA. 1985. Purification of *Legionella pneumophila* major outer membrane protein and demonstration that it is a porin. J Bacteriol 162(1):85-91.

Gagnon E, Duclos S, Rondeau C, Chevet E, Cameron PH, Steele-Mortimer O, Paiement J, Bergeron JJ, Desjardins M. 2002. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell 110(1):119-31.

Gao LY, Kwaik YA. 2000. The mechanism of killing and exiting the protozoan host *Acanthamoeba polyphaga* by *Legionella pneumophila*. Environ Microbiol 2(1):79-90.

Garcia-Vidal C, Carratala J. 2006. Current clinical management of Legionnaires' disease. Expert Rev Anti Infect Ther 4(6):995-1004.

Garin J, Diez R, Kieffer S, Dermine JF, Duclos S, Gagnon E, Sadoul R, Rondeau C, Desjardins M. 2001. The phagosome proteome: insight into phagosome functions. J Cell Biol 152(1):165-80.

Glockner G, Albert-Weissenberger C, Weinmann E, Jacobi S, Schunder E, Steinert M, Hacker J, Heuner K. 2007. Identification and characterization of a new conjugation/type IVA secretion system (trb/tra) of *Legionella pneumophila* Corby localized on two mobile genomic islands. Int J Med Microbiol.

Goldberg MB. 2001. Actin-based motility of intracellular microbial pathogens. Microbiol Mol Biol Rev 65(4):595-626, table of contents.

Goldsbrough AP, Bulleid NJ, Freedman RB, Flavell RB. 1989. Conformational differences between two wheat (*Triticum aestivum*) 'high-molecular-weight' glutenin subunits are due to a short region containing six amino acid differences. Biochem J 263(3):837-42.

Gotthardt D, Blancheteau V, Bosserhoff A, Ruppert T, Delorenzi M, Soldati T. 2006a. Proteomics fingerprinting of phagosome maturation and evidence for the role of a Galpha during uptake. Mol Cell Proteomics 5(12):2228-43.

Gotthardt D, Dieckmann R, Blancheteau V, Kistler C, Reichardt F, Soldati T. 2006b. Preparation of intact, highly purified phagosomes from *Dictyostelium*. Methods Mol Biol 346:439-48.

Gotthardt D, Warnatz HJ, Henschel O, Bruckert F, Schleicher M, Soldati T. 2002. High-resolution dissection of phagosome maturation reveals distinct membrane trafficking phases. Mol Biol Cell 13(10):3508-20.

Grogan A, Reeves E, Keep N, Wientjes F, Totty NF, Burlingame AL, Hsuan JJ, Segal AW. 1997. Cytosolic phox proteins interact with and regulate the assembly of coronin in neutrophils. J Cell Sci 110 (Pt 24):3071-81.

Hägele S. 2002. Wirtsspezifitat der Gattung *Legionella* und Etablierung von *Dictyostelium discoideum* als Wirtsmodel. Wuerzburg: Universitet Wuerzburg.

Hägele S, Kohler R, Merkert H, Schleicher M, Hacker J, Steinert M. 2000. *Dictyostelium discoideum*: a new host model system for intracellular pathogens of the genus *Legionella*. Cell Microbiol 2(2):165-71.

Hales LM, Shuman HA. 1999a. *Legionella pneumophila* contains a type II general secretion pathway required for growth in amoebae as well as for secretion of the Msp protease. Infect Immun 67(7):3662-6.

Hales LM, Shuman HA. 1999b. The *Legionella pneumophila* rpoS gene is required for growth within Acanthamoeba castellanii. J Bacteriol 181(16):4879-89.

Hall A. 1998. Rho GTPases and the actin cytoskeleton. Science 279(5350):509-14.

Harb OS, Gao LY, Abu Kwaik Y. 2000. From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. Environ Microbiol 2(3):251-65.

Harb OS, Venkataraman C, Haack BJ, Gao LY, Kwaik YA. 1998. Heterogeneity in the attachment and uptake mechanisms of the Legionnaires' disease bacterium, *Legionella pneumophila*, by protozoan hosts. Appl Environ Microbiol 64(1):126-32.

Harricane MC, Caron E, Porte F, Liautard JP. 1996. Distribution of annexin I during non-pathogen or pathogen phagocytosis by confocal imaging and immunogold electron microscopy. Cell Biol Int 20(3):193-203.

Harrison RE, Bucci C, Vieira OV, Schroer TA, Grinstein S. 2003. Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP. Mol Cell Biol 23(18):6494-506.

Hart MJ, Maru Y, Leonard D, Witte ON, Evans T, Cerione RA. 1992. A GDP dissociation inhibitor that serves as a GTPase inhibitor for the Ras-like protein CDC42Hs. Science 258(5083):812-5.

Hart PD, Young MR. 1991. Ammonium chloride, an inhibitor of phagosome-lysosome fusion in macrophages, concurrently induces phagosome-endosome fusion, and opens a novel pathway: studies of a pathogenic mycobacterium and a nonpathogenic yeast. J Exp Med 174(4):881-9.

Hatakeyama S, Matsumoto M, Yada M, Nakayama KI. 2004. Interaction of U-box-type ubiquitin-protein ligases (E3s) with molecular chaperones. Genes Cells 9(6):533-48.

Hell W, Essig A, Bohnet S, Gatermann S, Marre R. 1993. Cleavage of tumor necrosis factor-alpha by *Legionella* exoprotease. Apmis 101(2):120-6.

Heuner K, Steinert M. 2003. The flagellum of *Legionella pneumophila* and its link to the expression of the virulent phenotype. Int J Med Microbiol 293(2-3):133-43.

Heuner R, Broich, Schunder, Bender, Higa, Fujita, Lorenz, Schmeck, Dje N'Guessan, Hippenstiel and Flieger. 2007. The cell-associated phospholipase A PlaB of *Legionella pneumophila* is an inducer of inflammation and virulence. in press.

Hilbi H. 2006. Modulation of phosphoinositide metabolism by pathogenic bacteria. Cell Microbiol 8(11):1697-706.

Hilbi H, Weber SS, Ragaz C, Nyfeler Y, Urwyler S. 2007. Environmental predators as models for bacterial pathogenesis. Environ Microbiol 9(3):563-75.

Horwitz MA. 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. J Exp Med 158(4):1319-31.

Houde M, Bertholet S, Gagnon E, Brunet S, Goyette G, Laplante A, Princiotta MF, Thibault P, Sacks D, Desjardins M. 2003. Phagosomes are competent organelles for antigen cross-presentation. Nature 425(6956):402-6.

Houliston RS, Liu C, Singh LM, Meiering EM. 2002. pH and urea dependence of amide hydrogen-deuterium exchange rates in the beta-trefoil protein hisactophilin.

Biochemistry 41(4):1182-94.

Humphries CL, Balcer HI, D'Agostino JL, Winsor B, Drubin DG, Barnes G, Andrews BJ, Goode BL. 2002. Direct regulation of Arp2/3 complex activity and function by the actin binding protein coronin. J Cell Biol 159(6):993-1004.

Imai K, Kijima T, Noda Y, Sutoh K, Yoda K, Adachi H. 2002. A Rho GDP-dissociation inhibitor is involved in cytokinesis of *Dictyostelium*. Biochem Biophys Res Commun 296(2):305-12.

Jacobi S, Heuner K. 2003. Description of a putative type I secretion system in *Legionella pneumophila*. Int J Med Microbiol 293(5):349-58.

James BW, Mauchline WS, Dennis PJ, Keevil CW, Wait R. 1999. Poly-3-hydroxybutyrate in *Legionella pneumophila*, an energy source for survival in low-nutrient environments. Appl Environ Microbiol 65(2):822-7.

Jonas D, Engels I, Daschner FD, Frank U. 2000. The effect of azithromycin on intracellular *Legionella pneumophila* in the Mono Mac 6 cell line at serum concentrations attainable in vivo. J Antimicrob Chemother 46(3):385-90.

Jutras I, Desjardins M. 2005. Phagocytosis: at the crossroads of innate and adaptive immunity. Annu Rev Cell Dev Biol 21:511-27.

Kagan JC, Roy CR. 2002. *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. Nat Cell Biol 4(12):945-54.

Kagan JC, Stein MP, Pypaert M, Roy CR. 2004. *Legionella* subvert the functions of Rab1 and Sec22b to create a replicative organelle. J Exp Med 199(9):1201-11.

Kalamidas SA, Kuehnel MP, Peyron P, Rybin V, Rauch S, Kotoulas OB, Houslay M, Hemmings BA, Gutierrez MG, Anes E and others. 2006. cAMP synthesis and degradation by phagosomes regulate actin assembly and fusion events: consequences for mycobacteria. J Cell Sci 119(Pt 17):3686-94.

Karimova G, Pidoux J, Ullmann A, Ladant D. 1998. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proc Natl Acad Sci U S A 95(10):5752-6.

Katz SM, Hashemi S. 1982. Electron microscopic examination of the inflammatory response to *Legionella pneumophila* in guinea pigs. Lab Invest 46(1):24-32.

King CH, Shotts EB, Jr., Wooley RE, Porter KG. 1988. Survival of coliforms and bacterial pathogens within protozoa during chlorination. Appl Environ Microbiol 54(12):3023-33.

Kjeken R, Egeberg M, Habermann A, Kuehnel M, Peyron P, Floetenmeyer M, Walther P, Jahraus A, Defacque H, Kuznetsov SA and others. 2004. Fusion between phagosomes, early and late endosomes: a role for actin in fusion between late, but not early endocytic organelles. Mol Biol Cell 15(1):345-58.

Knaus UG, Heyworth PG, Evans T, Curnutte JT, Bokoch GM. 1991. Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac 2. Science 254(5037):1512-5.

Kuhlmann M, Popova B, Nellen W. 2006. RNA interference and antisense-mediated gene silencing in *Dictyostelium*. Methods Mol Biol 346:211-26.

Kuspa A, Loomis WF. 1992. Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. Proc Natl Acad Sci U S A 89(18):8803-7.

Laguna RK, Creasey EA, Li Z, Valtz N, Isberg RR. 2006. A *Legionella pneumophila*-translocated substrate that is required for growth within macrophages and protection from host cell death. Proc Natl Acad Sci U S A 103(49):18745-50.

Lemmon MA. 2003. Phosphoinositide recognition domains. Traffic 4(4):201-13.

Li Z, Solomon JM, Isberg RR. 2005. *Dictyostelium discoideum* strains lacking the RtoA protein are defective for maturation of the *Legionella pneumophila* replication vacuole. Cell Microbiol 7(3):431-42.

Lilic M, Galkin VE, Orlova A, VanLoock MS, Egelman EH, Stebbins CE. 2003. *Salmonella* SipA polymerizes actin by stapling filaments with nonglobular protein arms. Science 301(5641):1918-21.

Loomis WF, Jr. 1971. Sensitivity of *Dictyostelium discoideum* to nucleic acid analogues. Exp Cell Res 64(2):484-6.

Lu H, Clarke M. 2005. Dynamic properties of *Legionella*-containing phagosomes in *Dictyostelium* amoebae. Cell Microbiol 7(7):995-1007.

Luhrmann A, Haas A. 2000. A method to purify bacteria-containing phagosomes from infected macrophages. Methods Cell Sci 22(4):329-41.

Lund M, Akesson T, Jonsson B. 2005. Enhanced protein adsorption due to charge regulation. Langmuir 21(18):8385-8.

Luo ZQ, Isberg RR. 2004. Multiple substrates of the *Legionella pneumophila* Dot/Icm system identified by interbacterial protein transfer. Proc Natl Acad Sci U S A 101(3):841-6.

Mandell LA, Bartlett JG, Dowell SF, File TM, Jr., Musher DM, Whitney C. 2003. Update of practice guidelines for the management of community-acquired pneumonia in immunocompetent adults. Clin Infect Dis 37(11):1405-33.

Marra A, Blander SJ, Horwitz MA, Shuman HA. 1992. Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. Proc Natl Acad Sci U S A 89(20):9607-11.

Mintz CS, Miller RD, Gutgsell NS, Malek T. 1993. *Legionella pneumophila* protease inactivates interleukin-2 and cleaves CD4 on human T cells. Infect Immun

61(8):3416-21.

Moffat JF, Edelstein PH, Regula DP, Jr., Cirillo JD, Tompkins LS. 1994. Effects of an isogenic Zn-metalloprotease-deficient mutant of *Legionella pneumophila* in a guinea-pig pneumonia model. Mol Microbiol 12(5):693-705.

Molmeret M, Zink SD, Han L, Abu-Zant A, Asari R, Bitar DM, Abu Kwaik Y. 2004. Activation of caspase-3 by the Dot/Icm virulence system is essential for arrested biogenesis of the *Legionella*-containing phagosome. Cell Microbiol 6(1):33-48.

Molofsky AB, Swanson MS. 2004. Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle. Mol Microbiol 53(1):29-40.

Muller-Taubenberger A, Lupas AN, Li H, Ecke M, Simmeth E, Gerisch G. 2001. Calreticulin and calnexin in the endoplasmic reticulum are important for phagocytosis. Embo J 20(23):6772-82.

Nagai H, Kagan JC, Zhu X, Kahn RA, Roy CR. 2002. A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. Science 295(5555):679-82.

Newton AC. 1995. Protein kinase C: structure, function, and regulation. J Biol Chem 270(48):28495-8.

Nishi T, Forgac M. 2002. The vacuolar (H+)-ATPases--nature's most versatile proton pumps. Nat Rev Mol Cell Biol 3(2):94-103.

Noegel AA, Schleicher M. 2000. The actin cytoskeleton of *Dictyostelium*: a story told by mutants. J Cell Sci 113 (Pt 5):759-66.

O'Day DH, Durston AJ. 1979. Evidence for chemotaxis during sexual development in *Dictyostelium discoideum*. Can J Microbiol 25(4):542-4.

Parish RW. 1975. Mitochondria and peroxisomes from the cellular slime mould *Dictyostelium discoideum*. Isolation techniques and urate oxidase association with peroxisomes. Eur J Biochem 58(2):523-31.

Payne NR, Horwitz MA. 1987. Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. J Exp Med 166(5):1377-89.

Peracino B, Wagner C, Balest A, Balbo A, Pergolizzi B, Noegel AA, Steinert M, Bozzaro S. 2006. Function and mechanism of action of *Dictyostelium* Nramp1 (Slc11a1) in bacterial infection. Traffic 7(1):22-38.

Pethe K, Swenson DL, Alonso S, Anderson J, Wang C, Russell DG. 2004. Isolation of *Mycobacterium tuberculosis* mutants defective in the arrest of phagosome maturation. Proc Natl Acad Sci U S A 101(37):13642-7.

Pittis MG, Muzzolin L, Giulianini PG, Garcia RC. 2003. *Mycobacteria*-containing phagosomes associate less annexins I, VI, VII and XI, but not II, concomitantly with a diminished phagolysosomal fusion. Eur J Cell Biol 82(1):9-17.

Pizarro-Cerda J, Meresse S, Parton RG, van der Goot G, Sola-Landa A, Lopez-Goni I, Moreno E, Gorvel JP. 1998. *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. Infect Immun 66(12):5711-24.

Pollard TD, Borisy GG. 2003. Cellular motility driven by assembly and disassembly of actin filaments. Cell 112(4):453-65.

Pope CD, Dhand L, Cianciotto NP. 1994. Random mutagenesis of *Legionella pneumophila* with mini-Tn10. FEMS Microbiol Lett 124(1):107-11.

Pukatzki S, Kessin RH, Mekalanos JJ. 2002. The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. Proc Natl Acad Sci U S A 99(5):3159-64.

Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, Heidelberg JF, Mekalanos JJ. 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proc Natl Acad Sci U S A 103(5):1528-33.

Quinn FD, Keen MG, Tompkins LS. 1989. Genetic, immunological, and cytotoxic comparisons of *Legionella* proteolytic activities. Infect Immun 57(9):2719-25.

Quinn FD, Tompkins LS. 1989. Analysis of a cloned sequence of *Legionella pneumophila* encoding a 38 kD metalloprotease possessing haemolytic and cytotoxic activities. Mol Microbiol 3(6):797-805.

Ramachandra L, Sramkoski RM, Canaday DH, Boom WH, Harding CV. 1998. Flow analysis of MHC molecules and other membrane proteins in isolated phagosomes. J Immunol Methods 213(1):53-71.

Raper KB, Smith NR. 1939. The Growth of *Dictyostelium discoideum* upon Pathogenic Bacteria. J Bacteriol 38(4):431-45.

Rechnitzer C, Diamant M, Pedersen BK. 1989. Inhibition of human natural killer cell activity by *Legionella pneumophila* protease. Eur J Clin Microbiol Infect Dis 8(11):989-92.

Robinson CG, Roy CR. 2006. Attachment and fusion of endoplasmic reticulum with vacuoles containing *Legionella pneumophila*. Cell Microbiol 8(5):793-805.

Rodriguez-Paris JM, Nolta KV, Steck TL. 1993. Characterization of lysosomes isolated from *Dictyostelium discoideum* by magnetic fractionation. J Biol Chem 268(12):9110-6.

Rossier O, Cianciotto NP. 2001. Type II protein secretion is a subset of the PilD-dependent processes that facilitate intracellular infection by *Legionella pneumophila*. Infect Immun 69(4):2092-8.

Rossier O, Starkenburg SR, Cianciotto NP. 2004. *Legionella pneumophila* type II protein secretion promotes virulence in the A/J mouse model of Legionnaires' disease

pneumonia. Infect Immun 72(1):310-21.

Roy CR, Berger KH, Isberg RR. 1998. *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. Mol Microbiol 28(3):663-74.

Rupper A, Cardelli J. 2001. Regulation of phagocytosis and endo-phagosomal trafficking pathways in *Dictyostelium discoideum*. Biochim Biophys Acta 1525(3):205-16.

Shohdy N, Efe JA, Emr SD, Shuman HA. 2005. Pathogen effector protein screening in yeast identifies *Legionella* factors that interfere with membrane trafficking. Proc Natl Acad Sci U S A 102(13):4866-71.

Skriwan C, Fajardo M, Hagele S, Horn M, Wagner M, Michel R, Krohne G, Schleicher M, Hacker J, Steinert M. 2002. Various bacterial pathogens and symbionts infect the amoeba *Dictyostelium discoideum*. Int J Med Microbiol 291(8):615-24.

Solomon JM, Isberg RR. 2000. Growth of *Legionella pneumophila* in *Dictyostelium discoideum*: a novel system for genetic analysis of host-pathogen interactions. Trends Microbiol 8(10):478-80.

Solomon JM, Leung GS, Isberg RR. 2003. Intracellular replication of *Mycobacterium marinum* within *Dictyostelium discoideum*: efficient replication in the absence of host coronin. Infect Immun 71(6):3578-86.

Solomon JM, Rupper A, Cardelli JA, Isberg RR. 2000. Intracellular growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of host-pathogen interactions. Infect Immun 68(5):2939-47.

Spalekova M, Danihel L. 1994. [Comparison of susceptibility to Legionnaires' disease in small laboratory animals]. Epidemiol Mikrobiol Imunol 43(2):75-8.

Steinert M, Heuner K. 2005. *Dictyostelium* as host model for pathogenesis. Cell Microbiol 7(3):307-14.

Stoeckelhuber M, Noegel AA, Eckerskorn C, Kohler J, Rieger D, Schleicher M. 1996. Structure/function studies on the pH-dependent actin-binding protein hisactophilin in *Dictyostelium* mutants. J Cell Sci 109 (Pt 7):1825-35.

Stout JE, Yu VL. 1997. Legionellosis. N Engl J Med 337(10):682-7.

Strasser JE, Newman SL, Ciraolo GM, Morris RE, Howell ML, Dean GE. 1999. Regulation of the macrophage vacuolar ATPase and phagosome-lysosome fusion by *Histoplasma capsulatum*. J Immunol 162(10):6148-54.

Sturgill-Koszycki S, Haddix PL, Russell DG. 1997. The interaction between *Mycobacterium* and the macrophage analyzed by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis 18(14):2558-65.

Sturgill-Koszycki S, Swanson MS. 2000. *Legionella pneumophila* replication vacuoles mature

into acidic, endocytic organelles. J Exp Med 192(9):1261-72.

Sultana H, Rivero F, Blau-Wasser R, Schwager S, Balbo A, Bozzaro S, Schleicher M, Noegel AA. 2005. Cyclase-associated protein is essential for the functioning of the endo-lysosomal system and provides a link to the actin cytoskeleton. Traffic 6(10):930-46.

Sussman R, Sussman M. 1967. Cultivation of *Dictyostelium discoideum* in axenic medium. Biochem Biophys Res Commun 29(1):53-5.

Swanson JA, Hoppe AD. 2004. The coordination of signaling during Fc receptor-mediated phagocytosis. J Leukoc Biol 76(6):1093-103.

Swanson MS, Hammer BK. 2000. *Legionella pneumophila* pathogesesis: a fateful journey from amoebae to macrophages. Annu Rev Microbiol 54:567-613.

Swanson MS, Isberg RR. 1995. Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. Infect Immun 63(9):3609-20.

Szeto L, Shuman HA. 1990. The *Legionella pneumophila* major secretory protein, a protease, is not required for intracellular growth or cell killing. Infect Immun 58(8):2585-92.

Tesh MJ, Morse SA, Miller RD. 1983. Intermediary metabolism in *Legionella pneumophila*: utilization of amino acids and other compounds as energy sources. J Bacteriol 154(3):1104-9.

Tezcan-Merdol D, Engstrand L, Rhen M. 2005. Salmonella enterica SpvB-mediated ADP-ribosylation as an activator for host cell actin degradation. Int J Med Microbiol 295(4):201-12.

Tilney LG, Harb OS, Connelly PS, Robinson CG, Roy CR. 2001. How the parasitic bacterium *Legionella pneumophila* modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. J Cell Sci 114(Pt 24):4637-50.

Tsukano H, Kura F, Inoue S, Sato S, Izumiya H, Yasuda T, Watanabe H. 1999. *Yersinia pseudotuberculosis* blocks the phagosomal acidification of B10.A mouse macrophages through the inhibition of vacuolar H(+)-ATPase activity. Microb Pathog 27(4):253-63.

Unal C, Steinert M. 2006. *Dictyostelium discoideum* as a model to study host-pathogen interactions. Methods Mol Biol 346:507-15.

Via LE, Deretic D, Ulmer RJ, Hibler NS, Huber LA, Deretic V. 1997. Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. J Biol Chem 272(20):13326-31.

Vieira OV, Botelho RJ, Rameh L, Brachmann SM, Matsuo T, Davidson HW, Schreiber A, Backer JM, Cantley LC, Grinstein S. 2001. Distinct roles of class I and class III phosphatidylinositol 3-kinases in phagosome formation and maturation. J Cell Biol 155(1):19-25.

Vogel JP, Isberg RR. 1999. Cell biology of *Legionella pneumophila*. Curr Opin Microbiol 2(1):30-4.

Wagner C, Khan AS, Kamphausen T, Schmausser B, Unal C, Lorenz U, Fischer G, Hacker J, Steinert M. 2007. Collagen binding protein Mip enables *Legionella pneumophila* to transmigrate through a barrier of NCI-H292 lung epithelial cells and extracellular matrix. Cell Microbiol 9(2):450-62.

Walburger A, Koul A, Ferrari G, Nguyen L, Prescianotto-Baschong C, Huygen K, Klebl B, Thompson C, Bacher G, Pieters J. 2004. Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. Science 304(5678):1800-4.

Watts DJ, Ashworth JM. 1970. Growth of myxameobae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. Biochem J 119(2):171-4.

Weber SS, Ragaz C, Reus K, Nyfeler Y, Hilbi H. 2006. *Legionella pneumophila* exploits PI(4)P to anchor secreted effector proteins to the replicative vacuole. PLoS Pathog 2(5):e46.

Welch RA. 1991. Pore-forming cytolysins of gram-negative bacteria. Mol Microbiol 5(3):521-8.

Wiater LA, Dunn K, Maxfield FR, Shuman HA. 1998. Early events in phagosome establishment are required for intracellular survival of *Legionella pneumophila*. Infect Immun 66(9):4450-60.

Yu VL, Greenberg RN, Zadeikis N, Stout JE, Khashab MM, Olson WH, Tennenberg AM. 2004. Levofloxacin efficacy in the treatment of community-acquired legionellosis. Chest 125(6):2135-9.

Zeichner SL. 1982. Isolation and characterization of phagosomes containing *Chlamydia psittaci* from L cells. Infect Immun 38(1):325-42.

Zischka H, Oehme F, Pintsch T, Ott A, Keller H, Kellermann J, Schuster SC. 1999. Rearrangement of cortex proteins constitutes an osmoprotective mechanism in *Dictyostelium*. Embo J 18(15):4241-9.

8 Supplementary materials

8.1 Abbreviations

µg	microgram
µl	microliter
µM	micromolar
µm	micrometer
a.a.	amino acid
mm	millimeter
mg	milligram
mM	millimolar
kDa	kilo Dalton
HRP	horseradish peroxidase
g	gram
TCA	trichloroacetic acid
EDTA	ethylene diamino tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
min	minute
DIG	digoxzgenin
DMF	dimethylformamide
TCEP	tris[2-carboxyethyl] phosphine
nmol	nanomolar
APS	ammonium persulfate
TEMED	N,N,N',N'-Tetramethylethylenediamine
IEF	isoelectric focusing
MALDI-TOF	matrix assisted laser desorption/ionization time-of-flight mass spectrometry
V	volume
LLAP	*Legionella*-like amoeba pathogen
BMMO	bone marrow macrophages
INT	iodonitro- tetrazolium formazan
tRNA	transfer ribonucleic acid
mRNA	messenger ribonucleic acid
Acc N.	accession number
phox	phagocyte oxidase
GFP	green fluorescent protein
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
NADPH	nicotinamide adenine dinucleotide phosphate
V-ATPase	vacuolar type H+-ATPase
BSA	bovine serum albumin
cAMP	cyclic AMP or 3'-5'-cyclic adenosine monophosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	ditiotreitol
EDTA	ethylenediamine tetraacetic acid
kb	kilo bases

LAMP	lysosomal associated membrane glycoprotein
LB	Luria-Bertani broth
LCP	*Legionella*-containing phagosome
mM	millimolar
MOI	multiple of infection
MOMP	major outer membrane protein
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDI	protein disulphide isomerase
PI(3)P	phosphoinositol-3-sphosphate
PLA	phospholipase A
PM	plasma membrane
REMI	restriction-enzyme-mediated integration
RNA	ribonucleic acid
RNAi	RNA-mediated interference
Rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNARES	soluble NSF attachment receptor
SSC	standart saline citrate
SSPE	saline-Sodium Phosphate-EDTA
T2SS	type 2 secretion system
T4SS	type 4 secretion system
TAE	tris-acetate-EDTA
U	units
UV	ultraviolet

8.2 Legends to figures and tables

8.3 Microarray design

Table 8-1 List of bacterial strains used for array design

Bacteria, strain	Complete sequence	Acc.N.	Reference
1. *Listeria monocytogenes* EGD-e	+	NC_003210	PMID: 11679669
2. *Escherichia coli* O157:H7 EDL933	+	NC_002655	PMID: 11206551
3. *Escherichia coli* CFT073	+	NC_004431	PMID: 12471157
4. Escherichia coli K12	+	NC_000913	PMID: 9278503
5. *Legionella pneumophila*	+	--	--
6. *Pseudomonas aeruginosa* PA01	+	NC_002516	PMID: 10984043
7. *Salmonella typhimurium* LT2	+	NC_003197	PMID: 11677609
8. *Shigella flexneri* M90T(pWR100 plasmid)	--	AL391753	PMID: 11115111
9. *Shigella flexneri* 2a str. 2457T	+	NC_004741	PMID: 12704152
10. *Vibrio cholerae* serotype O1 biotype ElTor strain N16961	+	TIGR	--
11. *Vibrio cholerae* serotype O139	--	TIGR	--
12. *Yersinia pestis* KIM	+	NC_004088	PMID: 12142430
13. Yersinia pestis strain CO92	+	NC_003143	PMID: 11586360
14. *Y. enterocolitica* 8081 (serotype 0:8) pYVe8081	+	NC_003222	PMID: 17173484

Table 8-2 List of bacterial virulence genes spotted onto array

***Listeria monocytogenes* EGDe**

AccN	Gene	Product (function)	Reference (PMID)
NC_003210	*flaA*	Flagellin protein	12454178
X15127	*hly*	Listeriolysin O	12454178
LA591975	*iap*	P60 extracellular protein, invasion associated protein Iap	12454178
AL591975	*inlA*	Internalin A	12454178
AL591975	*inlB*	Internalin B	12454178
AL591977	*clpE*	ATP-dependent protease	12454178
AL591974	*plcB*	Phospholipase C	12454178

***Escherichia coli* O157:H7 EDL933**

AccN	Gene	Product (function)	Reference (PMID)
NC_002655	*stx1A*	Shiga-like toxin 1 subunit A encoded within prophage CP-933V	PMID: 11206551
NC_002655	*stx1B*	Shiga-like toxin 1 subunit B encoded within prophage CP-933V"	PMID: 11206551
AAG55587	*stx2A*	Shiga-like toxin II subunit A encoded by bacteriophage BP-933W	PMID: 11206551
AAG55588	*stx2B*	Shiga-like toxin II subunit B encoded by bacteriophage BP-933W	AAG55588
AE005191	*fhuA*	Outer membrane protein receptor for ferrichrome, colicin M, and phages T1, T5, and phi80	PMID: 11206551
AAG58823	*eae*	Intimin adherence protein	PMID: 11206551
NC_002655	*fimA*	Major type 1 subunit fimbrin (pilin)	PMID: 11206551

***Escherichia coli* CFT073**

AccN	Gene	Product (function)	Reference (PMID)
NC_004431	***focA*** (F1C gene)	F1C major fimbrial subunit	PMID: 12471157
AE016766	*hlyA*	A-hemolysin	PMID: 12471157
NC_004431	*papA*	Fimbrial protein	PMID: 12471157
AE016768	*chuA*	Outer membrane heme/hemoglobin receptor	PMID: 12471157
AE016766	*iucC*	Aeribactin, sideroohore biosynthesis protein	PMID: 12471157
NC_004431	*hlyC*	Hemolysin C	PMID: 12471157

***Escherichia coli* K-12 , sub.strain MG1655**

AccN	Gene	Product (function)	Reference (PMID)
AE000353	*emrA*	Multidrug resistance secretion protein	PMID: 9278503
NC_000913	*tonB*	Uptake of iron, cyanocobalimin; sensitivity to phages, colicins	PMID: 9278503

***Pseudomonas aeruginosa* PA01**

AccN	Gene	Product (function)	Reference (PMID)
AE004824	*cupB5*	Adhesive protein	PMID: 10984043
AE004540	*fliC*	Flagellin type B .Alt protein name: flagellar filament protein	PMID: 10984043
AE004574	*flhA*	Flagellar biosynthesis protein FlhA	PMID: 10984043
AE004544	*toxA*	Exotoxin A precursor	PMID: 10984043
AE004444	*exoT*	Exoenzyme T(Type III Secretion)	PMID: 10984043
AE004801	*exoS*	Exoenzyme S (Type III Secretion)	PMID: 10984043
AE004645	*exoY*	Exoenzyme S (Type III Secretion)	PMID: 10984043
AE004597	*pcrD*	Protein secretion/export type III secretory apparatus (Type III Secretion)	PMID: 10984043
AE004597	*popN*	Outer membrane protein(Type III Secretion)	PMID: 10984043
AE004597	*pcrG*	Regulator in type III secretion	PMID: 10984043
AE004597	*pcrV*	Type III secretion protein PcrV	PMID: 10984043
AE004882	*hitB*	Iron (III)-transport system permease HitB	PMID: 10984043

***Salmonella typhimurium* LT2**

AccN	Gene	Product (function)	Reference (PMID)
AE008777	*tonB*	Energy transducer; uptake of iron, cyanocobalimin; sensitivity to phages, colicins	PMID: 11677609
AE008752	*fhuE*	Outer membrane receptor for Fe(III)-coprogen	PMID: 11677609
NC_003197	*sipA*	Cell invasion protein	PMID: 11677609
AE008831	*sipB*	Cell invasion protein	PMID: 11677609
AE008751	*flgN*	Flagellar biosynthesis protein	PMID: 11677609
NC_003197	*flgK*	Flagellar biosynthesis, hook-filament junction protein	PMID: 11677609
AE008787	*fliC*	Flagellar biosynthesis; flagellin	PMID: 11677609

AE008834	*sopD*	Secreted protein in the Sop family; transferred to eukaryotic cells	PMID: 11677609
NP_461813	*invJ*	Surface presentation of antigens; secretory proteins	PMID: 11677609

***Shigella flexneri* strainM90T, virulence plasmid pWR100**

AccN	Gene	Product (function)	Reference (PMID)
AL391753	*ipaA*	Secreted by the Mxi-Spa machinery, modulates entry of bacteria into epithelial cells	PMID: 11115111 PMID: 12753186
AL391753	*ipaB*	Secreted by the Mxi-Spa secretion machinery, required for entry into epithelial cells	PMID: 11115111 PMID: 12753186
AL391753	*ipaC*	Secreted by the Mxi-Spa secretion machinery, required for entry into epithelial cells	PMID: 11115111 PMID: 12753186
AL391753	*ipaD*	Secreted by the Mxi-Spa machinery, required for entry of bacteria into epithelial cells	PMID: 11115111 PMID: 12753186
AL391753	*virB*	Transcriptional activator required for tanscription of the ipa, mxi, and spa operons	PMID: 11115111
AL391753	*icsB*	Required at the postinvasion stage of Shigella pathogenicity	PMID: 12753186

***Shigella flexneri* 2a str. 2457T (genomic DNA)**

AccN	Gene	Product (function)	Reference (PMID)
NC_004741	*sitA*	Iron uptake	PMID: 12704152
AE016991	*iucD*	Iron uptake	PMID: 12704152
AE016992	*feoB*	Ferrous iron transport protein B	PMID: 12704152

***Vibrio cholerae* str.16961, O1 biotype: E1 Tor**

Acc.N	TIGR lokus name	Gene	Product (function)	Reference (PMID)
AE004112	VC0228	*wavD*	Hypothetical protein	PMID: 10952301
AE004112	VC0235	*wavJ*	Lipopolysaccharide biosynthesis protein, putative	PMID: 10952301
AE004168	VC0828	*tcpA*	Toxin co-regulated pilin	PMID: 10952301
AE004224	VC1457	*ctxA*	Cholera enterotoxin, A subunit	PMID: 10952301
AE004224	VC1456	*ctxB*	Cholera enterotoxin, B subunit	PMID: 10952301
AE004113	VC0245	*rfbG*	RfbG protein	PMID: 10952301

***Yersinia pestis* CO92, pPCP1**

AccN	Gene	Product (function)	Reference (PMID)
AJ414145	*iucC*	Siderophore biosynthesis protein IucC	PMID: 11586360
AL109969	*pla*	Plasminogen activator	PMID: 11586360

***Y. enterocolitica* 8081 (serotype 0:8) pYVe8081**

AccN	Gene	Product (function)	Reference (PMID)
NC_005017	*yopE(e)*	Cytotoxin	PMID: 11402007
NC_005017	*yopH(e)*	Inhibition of phagocytosis, Yop effector YopH	PMID: 11402007
NC_005017	*yopM(e)*	Inhibition of phagocytosis	PMID: 11402007
NC_005017	*yopO(e)*	Protein kinase	PMID: 11402007
NC_005017	*yopD(e)*	Involved in the translocation process	PMID: 11402007
NC_005017	*yopB(e)*	Pore-forming translocase	PMID: 11402007
Sanger	*invA(ch)*	In vitro mammalian cells invasion	PMID: 11402007
Sanger	*ail(ch)*	Attachment invasion locus protein	PMID: 11402007
Sanger	*myfA(ch)*	Fimbriae subunit	PMID: 11402007
Sanger	*yst*	Enterotoxin	PMID: 11402007
NC_005017	*yadA(ch)*	Yersinia adhesin	PMID: 11402007

Table 8-3List of virulence genes primers spotted onto array

Strain	Acc.N	Name of gene	Name of primers	Size of fragment (bp)	5‘-3‘sequence of primer	GC(%)	N	Tm
Escherichia coli CFT073	AE016768	*chuA*	chuA_L chuA_R	405	GGTACCGGACGAACCAACG GGTTTCACCATTGCTCTGGC	63.2 55.0	19 20	55.0 54.4
	NC_004431	*focA(F1C gene)*	focA_L focA_R	450	ACCCTGGGTGTTGCGACAA GGCCTGTCCAGTTGCGTAGTA	57.9 57.1	19 21	55.9 54.3
	AE016766	*hlyA*	hlyA_L hlyA_R	599	CCTTGTCAGGACGGCAGATG CGGTATCTGCATCTGCATTGC	60.0 52.4	20 21	55.3 55.6
	NC_004431	*hlyC*	hlyC_L hlyC_R	447	GCTTTTTTTTACCTGCCACCG CGAAAGGAGCAATCCAGTCAA	47.6 47.6	21 21	55.5 54.3
	AE016766	*iucC*	iucC_L iucC_R	412	AGCTAAAGCCGGTGCTGTCA GGCAGCCAGTTGTGATCCAG	55.0 60.0	20 20	55.0 55.3
	NC_004431	*papA*	papA_L papA_R	424	GGTTATTGCCGGTGCGGTA TGTTGCTGTCGCACCATCAA	57.9 50.0	19 20	55.7 55.7
Escherichia coli O157:H7 EDL933	AAG55588	*stx2B*	Stx2B_L stx2B_R	204	GCTTCTGTTAATGCAATGGCG TCCGGAGCCTGATTCACA	47.6 55.6	21 18	55.0 51.8
	AAG55587	*stx2A*	Stx2A_L stx2A_R	418	ATCTCAGGGGACCACATCGG GCGTAAGGCTTCTGCTGTGAC	60.0 57.1	20 21	55.8 54.2
	NC_002655	*stx1A*	Stx1A_L stx1A_R	326	GATTCGCTGAATGTCATTCGC ATCCCTGCAACACGCTGTAAC	47.6 52.4	21 21	54.7 54.1
	NC_002655	*stx1B*	Stx1B_L stx1B_R	210	CATTTTTTTCAGCAAGTGCGC CCCCTCCATTATGACAGGCA	42.9 55.0	21 20	55.1 54.6
	AE005191	*fhuA*	fhuA_L fhuA_R	430	CAGAAGGCCAAAGCCAGAATAA TCCGGCTCGTTCTGGAAGTAA	45.5 52.4	22 21	55.2 56.2
	AAG58823	*Eae*	Eae_L eae_R	415	CAGTCGCGATCTCTGAACGG ACTCATGCCAGCCGCTCAT	60.0 57.9	20 19	55.6 55.4

	Acc.N	*Name of gene*	Name of primers	Size of fragment(bp)	5‘-3‘sequence of primer	GC(%)	N	Tm
	NC_002655	*fimA*	fimA_L fimA_R	417	CTGGCAATCGTTGTTCTGTCG TTGCTCACTGAATGTCGCACC	52.4 52.4	21 21	55.6 56.0
E.coli K12	AE000353	*emrA*	emrA_L emrA_R	424	TTTTTGGTACTGCGTCACTTCG TGGCATTGTATTGTTGAATCGC	45.5 40.9	22 22	54.6 55.1
	NC_000913	*tonB*	tonB_L tonB_R	409	CCTTGATTTACCTCGCCGC TGCTGTACTTGATGTCAGGCGT	57.9 50.0	19 22	54.7 54.8
Listeria monocytogenes EGD-e	AL591977	*clpE*	clpE_L clpE_R	367	ATGGCAAGCGTGTGGAAATG ACACCTGGTTCACCGATTAGGAC	50.0 52.2	20 23	55.7 55.8
	NC_003210	*flaA*	flaA_L flaA_R	412	TAACAGTTCTCTTGATGACGCTGC TGTTCCAGCAGAAAGACCTTTAGC	45.8 45.8	24 24	55.3 55.9
	X15127	*hly*	Hly_L hly_R	597	AGCATCTCCGCCTGCAAGT TCACTGATTGCGCCGAAGTT	57.9 50.0	19 20	54.7 55.6
	LA591975	*iap*	Iap_L iap_R	533	CGAATCTAACGGCTGGCACA CTGTTTGTTGTTGCGTTGCTGT	55.0 45.5	20 22	56.1 55.3
	AL591975	*inlA*	inlA_L inlA_R	416	CCTAGCAGGTTTAACCGCACTC CGCCATCGCTAATAGTAGCTGG	54.5 54.5	22 22	54.8 55.8
	AL591975	*inlB*	inlB_L inlB_R	434	GTGATATTGTGCCACTTGCAGG CCGCGTCCCTGCTTCTACTT	50.0 60.0	22 20	54.6 55.9
	AL591974	*plcB*	plcB_L plcB_R	539	CCGCAGCTCCGCATGATAT TGCTACCATGTCTTCCGTTGC	57.9 52.4	19 21	55.9 55.1
P.aeruginosa PA01	AE004824	*cupB5*	cupB5_L cupB5_R	620	GAACACGCTCGCCAGCAAT GATTGCCGATGCTGTTGCC	57.9 57.9	19 19	56.0 56.2
	AE004801	*exoS*	exoS_L exoS_R	407	GTCCCTTCGTGGCGATCAT TGCGTCGAGCACCAGTTGA	57.9 57.9	19 19	

	Acc.N	*Name of gene*	Name of primers	Size of fragment(bp)	5‘-3’sequence of primer	GC(%)	N	Tm
Pseudomonas aeruginosa PA01	AE004444	*exoT*	exoT_L exoT_R	443	CCGCCGAGATCAAGCAGAT GAAGTGCTCCACCAGGCCAT	57.9 60.0	19 20	55.0 56.2
	AE004645	*exoY*	exoY_L exoY_R	417	CATCTCTCGGCAACGCCTT TTGAGGGCGTCCACCAGTT	57.9 57.9	19 19	55.1 55.1
	AE004574	*flhA*	flhA_L flhA_R	552	GCTTCACCCTGGATGCCAT CGACCTGCTTGGCCTTGTT	57.9 57.9	19 19	54.5 55.0
	AE004540	*fliC*	fliC_L fliC_R	430	CCCTGCAGAAAGAAGTCGCTG AAGTTCAGCGAACCACCGG	57.1 57.9	21 19	56.5 54.9
	AE004882	*hitB*	hitB_L hitB_R	543	GCTGAGCCCGATGTACGAAG AGCCAATAACCGAGCATCACC	60.0 52.4	20 21	54.8 55.5
	AE004597	*pcrD*	pcrD_L pcrD_R	451	TTTTCCCTGGATGCCATGC CGAACAGCACCAGCAACAGG	52.6 60.0	19 20	55.6 56.5
	AE004597	*pcrG*	pcrG_L pcrG_R	296	TGGGCGACATGAACGAATACA TCAGATCAACAAGCCACGCAT	47.6 47.6	21 21	56.2 55.5
	AE004597	*pcrV*	pcrV_L pcrV_R	453	CAGAAACCTTAATGCCGCTCG GCGTTGATCTGCGACTGGAT	52.4 55.0	21 20	56.4 55.0
	AE004597	*popN*	popN_L popN_R	509	ATCCTCCAGAGTTCCTCCGC AGGCCGAGTTCGATCTCCAG	60.0 60.0	20 20	54.5 55.6
	AE004544	*toxA*	toxA_L toxA_R	547	CGCTGCAACCTCGACGATA GATAGTTGCGCTCCAGCAGG	57.9 60.0	19 20	54.6 54.9
S. typhimurium LT2	AE008777	*tonB*	tonB_L tonB_R	425	ACCCTTGATTTACCTCGTCGC GATGCGGTGCTGCTTGTTG	52.4 57.9	21 19	54.7 54.9
	AE008752	*fhuE*	fhuE_L fhuE_R	489	CGGGACCGGTAATCCTTCTG TCAGTATGGGTCGCATTCAGG	60.0 52.4	20 21	55.7 54.9

	Acc.N	Name of gene	Name of primers	Size of fragment(bp)	5‘-3’sequence of primer	GC(%)	N	Tm
Salmonella typhimurium LT2	NC_003197	*sipA*	sipA_L sipA_R	411	GTTGAGGCGCTTGATATGTGC CGAATGGTGTGACTCGCCA	52.4 57.9	21 19	54.8 55.0
	AE008831	*sipB*	sipB_L sipB_R	412	CCGGCTATGCACAAGCTGA CATCCCATAATGCGGTTCGT	57.9 50.0	19 20	54.6 54.7
	AE008751	*flgN*	flgN_L flgN_R	342	TGACCAGATGACCACCGTCC TACAAGGTCGGCTCCTGGTG	60.0 60.0	20 20	55.2 54.2
	NC_003197	*flgK*	flgK_L flgK_R	485	GCTCACTGTCTGGTTCGTTGC GGCGGCCTCATCGACATAA	57.1 57.9	21 19	54.7 55.7
	AE008787	*fliC(sal)*	fliC(sal)_L fliC(sal)_R	458	TGACCTCGACTCCATCCAGG CAAGAGTCACCTCACCGTTCG	60.0 57.1	20 21	54.6 54.6
	AE008834	*sopD*	sopD_L sopD_R	475	CTGCCCGGCTCATCAAGAT GCACGGATGCATCATGCT	57.9 55.6	19 18	54.9 52.3
	NP_461813	*invJ*	invJ_L invJ_R	480	CGTTATTACTGGCGGCATGG GTTGCAGCGGAAGCTGAGAA	55.0 55.0	20 20	55.5 55.5
Shigella flexneri 2a str. 2457T	AE016992	*feoB*	feoB_L feoB_R	458	GTACCTGACGCTGCAACTGCT TGGCAGCAATGCACTGGTAAC	57.1 52.4	21 21	54.5 56.0
	AE016991	*iucD*	iucD _L iucD _R	441	ATCGCTTCCTTACAAGCAGGC CATCAGCAAAAGCAGCCTCAT	52.4 47.6	21 21	55.2 54.5
	NC_004741	*sitA*	sitA _L sitA _R	540	AAAAACGTGGCTGGAGATGCT CGGTGTTCCTTGTTGATCGG	47.6 55.0	21 20	55.3 55.2
Sh. flexneri pWR100	AL391753	*virB*	virB _L virB _R	432	TGAAATTCTGGATGGCACTCG GGGGGCAGATTTGTATCAAGG	47.6 52.4	21 21	55.0 54.9
	AL391753	*icsB*	icsB _L icsB _R	445	ACAGCCGAAAGACTTGATGCA TGAGACTTTCAATGCGTTGCC	47.6 47.6	21 21	54.8 55.2

	Acc.N	Name of gene	Name of primers	Size of fragment(bp)	5‘-3’sequence of primer	GC(%)	N	Tm
Shigella flexneri M90T (pWR100 plasmid)	*AL391753*	*ipaA*	ipaA _L ipaA _R	553	AGCCGTGTTTCCTGAGCTGTT GGGGGTGGTTGAAGAGTTCTG	52.4 57.1	21 21	55.4 54.8
	AL391753	*ipaB*	ipaB _L ipaB _R	496	ATGCAAGTTCCCAACTAACGCT TGGGTTGATAGCTGTTCAGCAG	45.5 50.0	22 22	54.6 54.5
	AL391753	*ipaC*	ipaC _L ipaC _R	320	AATTATCAGCAGATTGCAGCGC GCATCGAACGCAATCAATGAC	45.5 47.6	22 21	56.1 55.4
	AL391753	*ipaD*	ipaD _L ipaD _R	455	CCCGAAAGAAGCCGAGCTT CCTCGCCATTTCCACCTAGATT	57.9 50.0	19 22	55.9 56.1
Vibrio cholerae serotype O1 biotype ElTor strain N16961	*AE004224* *(VC1457)*	*ctxA*	ctxA _L ctxA _R	438	AGCAGTCAGGTGGTCTTATGCC CATAACCATCTGCTGCTGGAGC	54.5 54.5	22 22	54.8 56.3
	AE004224 *(VC1456)*	*ctxB*	ctxB_L ctxB _R	321	CAGCATATGCACATGGAACACC CCATACTAATTGCGGCAATCG	50.0 47.6	22 21	55.2 54.8
	AE004113 *(VC0245)*	*rfbG*	rfbG _L rfbG _R	488	TTGCAAAACTGCCAATTATCCC TTCAAATGCCGCCTTCTTAGAG	40.9 45.5	22 22	55.6 55.3
	AE004168 *(VC0828)*	*tcpA*	tcpA _L tcpA _R	408	TATTATGGGTGTGGTCTCAGCG AGATCAGCGACAGCAGCGA	50.0 57.9	22 19	54.1 54.3
	AE004112 *(VC0228)*	*wavD*	wavD _L wavD _R	484	CAACTGTTTCTGGATTACGCCA CCAAGTTCATCTCCGTCAACAA	45.5 45.5	22 22	54.3 54.1
	AE004112 *(VC0235)*	*wavJ*	wavJ _L wavJ _R	594	CCCTCAGACCTAGCGGACAA CGCTTTCGCAATGAAGGTG	60.0 52.6	20 19	54.2 54.2
Y. pestis CO92	*AJ414145*	iucC	*iucC_L* *iucC_R*	416	CTTAACCGCCCTGCAACACTA GGATGCCACGATAACAAGAGGT	52.4 50.0	21 22	54.5 54.6

	AL109969	*Pla*	pla_L pla_R	509	TGGCTTCCGGGTCAGGTAATA TTCCGCAAAGACTTTGGCA	52.4 47.4	21 19	55.9 54.2
Y. enterocolitica 8081 (serotype 0:8) pYVe8081	*Sanger*	*ail(ch)*	ail_L ail_R	355	ATGTGTACGCTGCGAGTGAAAGT GCCATTGACGTCTTACTTGCACT	47.8 47.8	23 23	55.5 55.0
	Sanger	*invA(ch)*	invA_L invA_R	416	GTAGCCGCAGATGTTGGTATGTC TGGACATCATCCTATGCGGC	52.5 55.0	23 20	55.2 55.6
	Sanger	*myfA(ch)*	myfA_L myfA_R	365	TGCTGTGTTAATGTTGGCTAGTGG AAGTCGCTTCCACACGCTCA	45.8 55.0	24 20	55.9 55.7
	NC_005017	*yadA(ch)*	yadA_L yadA_R	429	CCTAGGCGGGACCAATGC CCAGCCGCAAGATGTGTCA	66.7 57.9	18 19	55.2 55.2
	NC_005017	*yopB(e)*	yopB_L yopB_R	505	AGCTCCCTGCACCACTAGCA TGGGATATCAGGCCATCTTCC	60.0 52.4	20 21	54.8 55.2
	NC_005017	*yopD(e)*	yopD_L yopD_R	434	GGGCAAAGCGGTGAGGTTA CGGCCGGCAATGTTACTGT	57.9 57.9	19 19	55.0 55.8
	NC_005017	*yopE(e)*	yopE_L yopE_R	510	CCGGCATCAGTGTCAGGATC AGTTCCCCACTGCGAGAAGG	60.0 60.0	20 20	55.1 55.5
	NC_005017	*yopH(e)*	yopH_L yopH_R	400	AAAGTGCTGTTGGAGGCTGC TGGCATTAAGATCGGCGC	55.0 55.6	20 18	54.4 54.5
	NC_005017	*yopM(e)*	yopM_L yopM_R	357	TCACTGGAATTTCTTGCTGCTG CGCTGGCAGTTCGATCAACT	45.5 55.0	22 20	54.6 55.2
	NC_005017	*yopO(e)*	yopO_L yopO_R	404	ATCTTGAAGGGATGCGAGTGG TGCCTTGGCAAGGTGATTG	52.4 52.6	21 19	55.5 54.3
	Sanger	*yst*	yst_L yst_R	174	CTTCATTTGGAGCATTCGGC AACCCGCACAGGCAGGATTG	50.0 60.0	20 20	54.7 59.4

Table 8-3 List of reporter and selection gene primers

Acc.N	Name of gene	Name of primers	Size of fragment(bp)	5‘-3‘sequence of primer	GC(%)	N	Tm
X65326	*Luc*	luc_L luc_R	285	GGATTCTAAAACGGATTACCAG AAACCGTGATGGAATGGAACA	40.9 42.9	22 21	49.4 54.1
NC_003213	*luxA*	luxA_L luxA_R	321	AGTTCCCGAAAATCCAACTGAA TGGTGGCATTCACGTATGAGTC	40.9 50.0	22 22	54.3 54.8
AY364166	*Gfp*	gfp_L gfp_R	380	ACTTTTTCAAGAGTGCCATGCC CGAAAGGGCAGATTGTGTGG	45.5 55.0	22 20	54.9 55.3
U50980	*phoA*	phoA_L phoA_R	336	GGAAATTACTGCCGCACGTAA CATTTGCGCGAAGTCACATG	47.6 50.0	21 20	54.5 55.0
AJ510163	*Yfp*	yfp_L yfp_R	341	GCTGACCCTGAAGTTCATCTGC CGGCCATGATATAGACGTTGTG	54.5 50.0	22 22	55.3 54.6
AY192024	*sacB*	sacB_L sacB_R	352	GTTTTATCAAAAGGTCGGCGAC GCCGGATGTATAATTGCCTTCA	45.5 45.5	22 22	54.5 55.6

Table 8-4 List of selected *Legionella* genes and their primers

Name of gene	Product or function	Name of primers	Size of fragment(bp)	5‘-3‘sequence of primer	N	Tm
flaA	Flagellin	flaA_L flaR_R	480	CATGATGCAAACATCGATCCA CTGCTACTTCTGTTCCTGTTG	21 21	55.9 57.9
fliA	Sigma factor 28	fliA_L fliA_R	400	AACGCATTGCACATCATCTGC ATAAGACATCATCGGTTACTC	21 21	57.9 54.0
flaR	Putative transcriptional regulator	flaR_L flaR_R	580	CAATTGTCGAATCTGGTGGAT TAGGCTCAATCTTGAGTTGATAT	21 23	55.9 55.3
fleQ	Bacterial enchancer binding protein	fleQ_L fleQ_R	490	GTGACAAGCTTCGGACTATTT GAAAGAGAATGTATATTGCGAG	21 22	55.9 54.7
rpoN	Sigma factor 54	rpoN_L rpoN _R	600	TGGTCAACATCTCACGTTAAC CCTAGCAACTCAATGTCTTCA	21 21	55.9 55.9
gacA	GacA regulatory protein	gacA_L gacA_R	470	ATTGTTGATGACCATGCATTGGT TTCATGCCGCTAGTAATCATC	23 21	57.1 55.9
csrA	Global regulator	csrA_L csrA_R	190	TTGACTCGGCGTATAGGTGAA CGATTTATACTGCTTGTTCCGAAT	21 24	57.9 57.6
fleS	Bacterial enchancer binding protein	fleS_L fleS_R	530	AAATCCTTCAGCTGAAGCGAT GTCGCATAAGACTCGATGTAT	21 21	55.9 55.9
pilR	Bacterial enchancer binding protein	pilR_L pilR_R	590	GTGCTTGTTATAGATGACGAA AAACTCTGACTCCATCAACTC	21 22	56.5 56.5
pilE	Type IV pilin	pilE_L pilE_R	450	ATGCTCAGTCATGTACATTTTATG GATTCCAGCATTCTGGTTGC	24 20	56.5 55.9
pilD	Type IV prepilin-like protein specific leader peptidase PilD	pilD_L pilD_R	480	AGAGCAATGCTGTGAGCTATT CCTACTTTACCTGTCATCAGA	21 21	55.9 55.9
fliM	Flagellar basal body gene	fliM_L fliM_R	450	GAGATCGATGCATTACTGGAT TAATAATCGACCAAGTCATACACA	21 24	55.9 55.9

flgB	Flagellar basal body gene	flgB_L flgB_R	540	GAAGTACTGACAGCAACTATG CGTCTTTATCAGCTATTGGAC	21 21	55.9 55.9
motA1	Flagellar motor protein	motA1_L motA1_R	500	GATTGTTGACAGGTTTTCTCGC CGAGATTCCTCATGACTTGAA	22 21	58.4 55.9
motA2	Flagellar motor protein	motA2_L motA2_R	440	ACTACTCTGTGTATTTGGTGG GATTCGATCTCCATATCCATC	21 21	55.9 55.9
rpoS	Control of stationary phase and stress regulated genes	rpoS_L rpoS_R	610	ATGCAAGATGATGAAGAGCCA CATGATCCAGTTTTTGCGTCA	21 21	55.9 55.9
plaB	Phospholipase A	plaB_L plaB_R	590	ATGATTGTTATCTTCGTCCATGG GTAGGAGTTCACTGCATCATA	23 21	57.1 55.9
plcA	Phospholipase C	plcA_L plcA_R	580	GCAATGAGCATTAGCTTGTTG CTGTTGCAGTGCGACATGG	21 19	55.9 58.8
lipB	Putative lipase	lipB_L lipB_R	680	TGTCAAGGCTTATGCTTACTC TTCTAAGAAGCTCAGCAGCACAAG	21 21	55.9 55.9
mspA	Zinc metalloprotease	mspA_L mspA_R	520	AGCTGTAGCTATAGCATTAGG AGGAATTCTATCGTCATGGATAA	21 23	55.9 55.3
lssD	Putative *hlyD* family secretion protein	lssD_L lssD_R	610	TGCCATTCTTGATGAAGTAACTAC TAAACTGGAGAACGAACTGTG	24 21	57.6 55.9
lssZ	Component of type I secretion system	lssZ_L lssZ_R	500	GGAATCAATGCATATCTTGGC GGATTAAACCGTAATGATTAGGGC	21 24	55.9 59.3
ompR	Outer membrane protein R	ompR_L ompR_R	450	TTGAAGACGATGAACTGCTTG ACATTGACGAGTACATCATCC	21 21	55.9 55.9
dotA	Dot/Icm effector protein	dotA_L dotA_R	420	TTTTCCTGCGCTTGCACT CGGTGGAGATAACTCAAGG	19 19	56.7 54.5

icmR	Required for macrophage killing	icmR_L icmR_R	350	ATACTGATGACAGTGCACGAA GATGATAATTTGAAACCACGTTC	21 23	55.9 55.3
fleN	Putative function	fleN_L fleN_R	540	TAGCCATAGCGTTATCTCAAC GTAATCTAACTGCACATCCAG	21 21	55.9 55.9
flgG	Flagellar basal body gene	flgG_L flgG_R	570	GCGAATAATCTTGCTAACGTC TACCGCTTGCTACAGTTTCC	21 20	55.9 57.3
traD	Putative type IVA secretion system	traD_L traD_R	--	GCTTATCATCACTTGCCCTTT GCAGAGATACACCACCAATCCGA	22 23	56.5 62.4
lvhB10	Putative type IVA secretion system	lvhB10_L lvhB10_R	710	GCAATCGGACTCAGGTTGCTA CTGCCAAAGCGCTCGAAGAAA	21 21	59.8 59.8
mip	Macrophage infectivity potentiator	mip_L mip_R	970	GATGAAATTGGTGACTGCGGCT CAATAGGTCCGCCAACGCTACGT	22 23	56.2 58.0

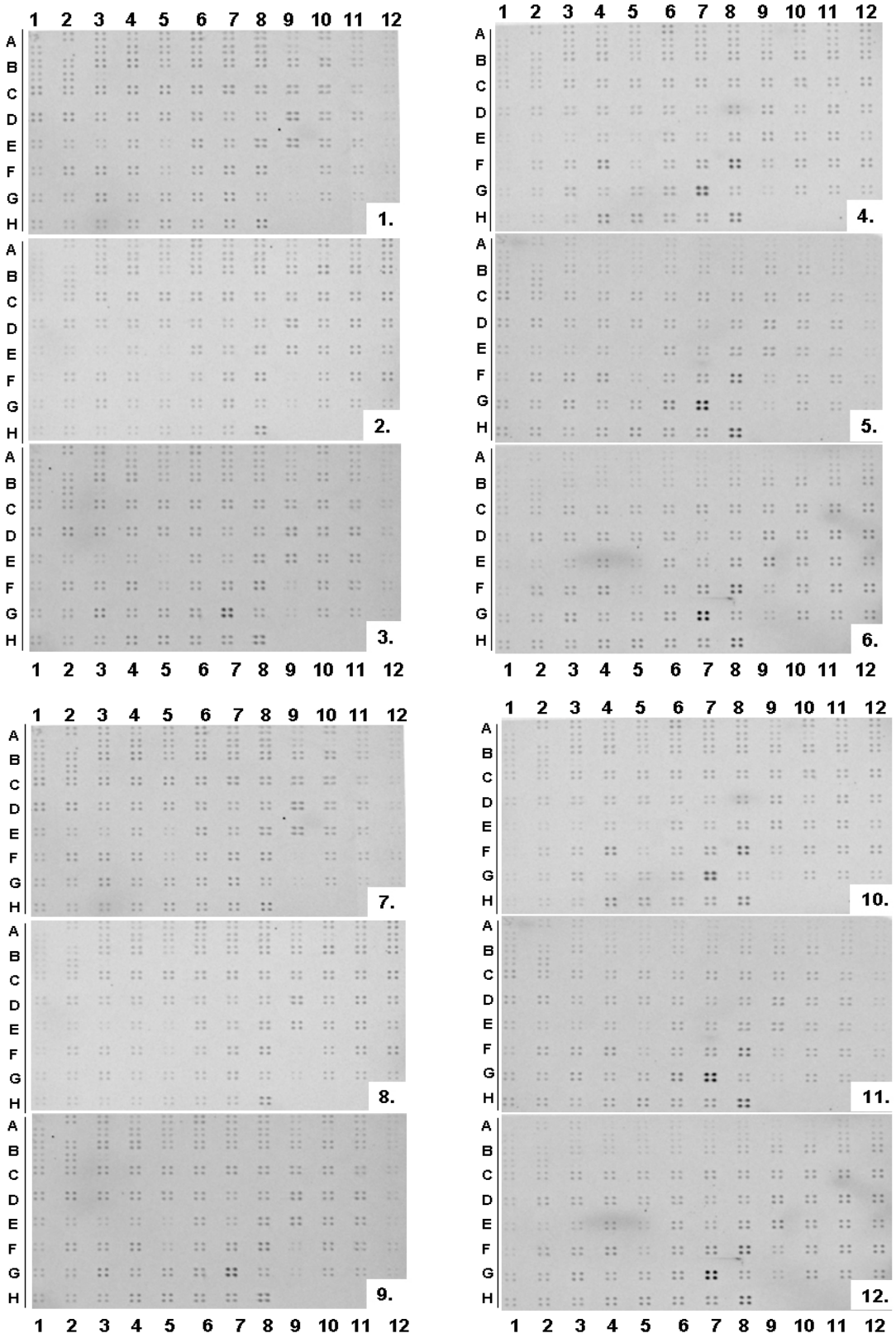


Fig. 8-1 Hybrydization of chromosomal DNA from different *Legionella* species with "pathoarray"
***L. pneumophila* Corby (1); *L. micdadei,* environment isolate (2); *L. micdadei*, patient isolate (3); *L. erythra* (4); *L. bozemanii* (5); *L. hackeliae* (6); *L. longbeacheae* (7); *L. pneumophila*, patient isolates (8, 9, 10); LLAP10 (11); *L. lythica* (12). Hybridization was performed under low stringency condition**

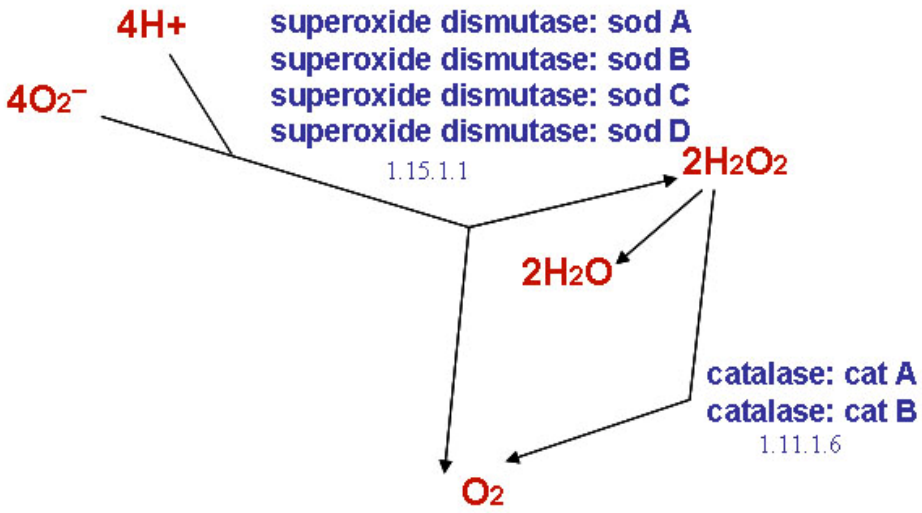
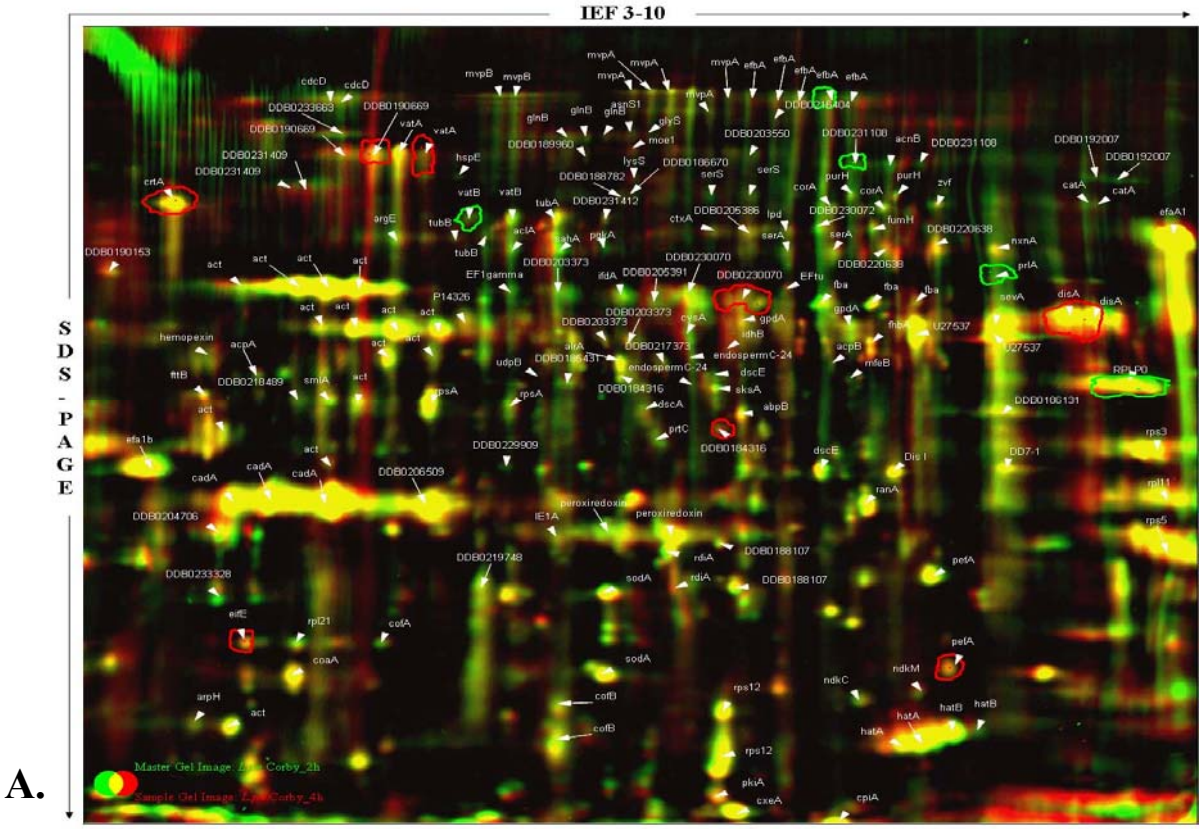
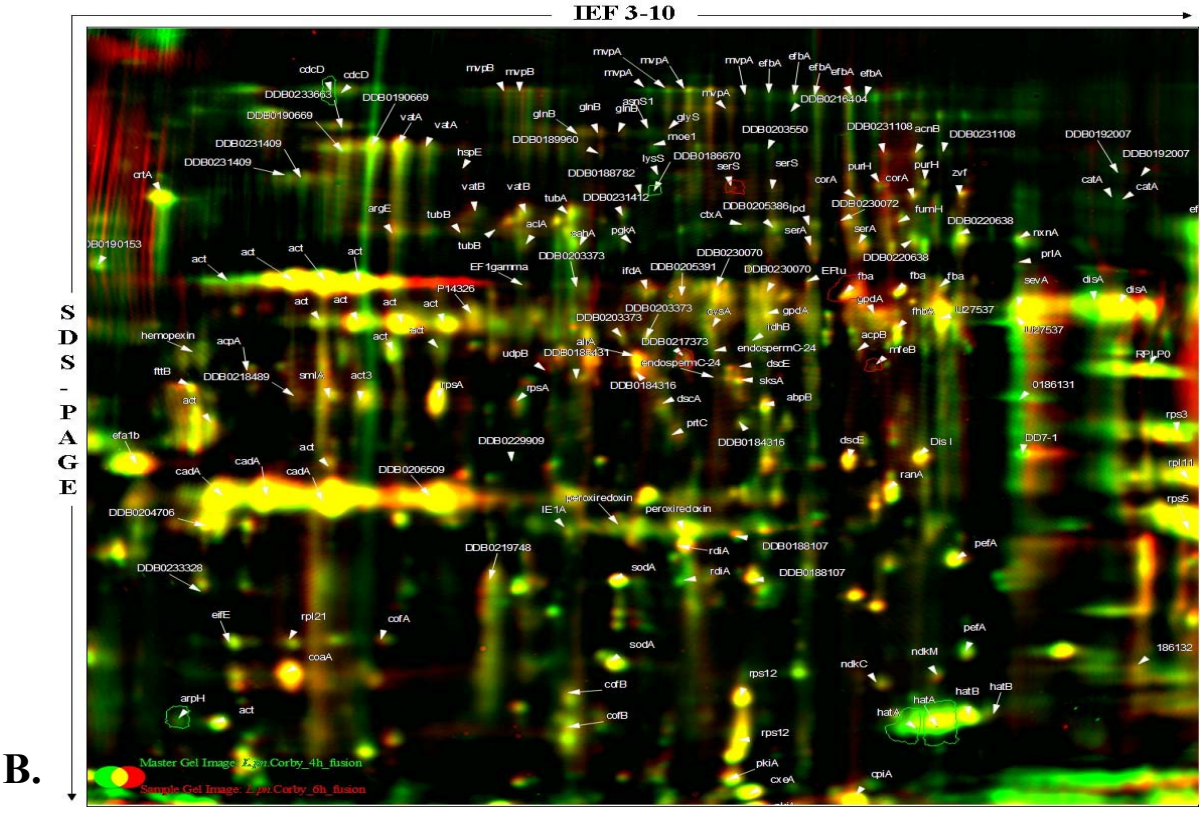


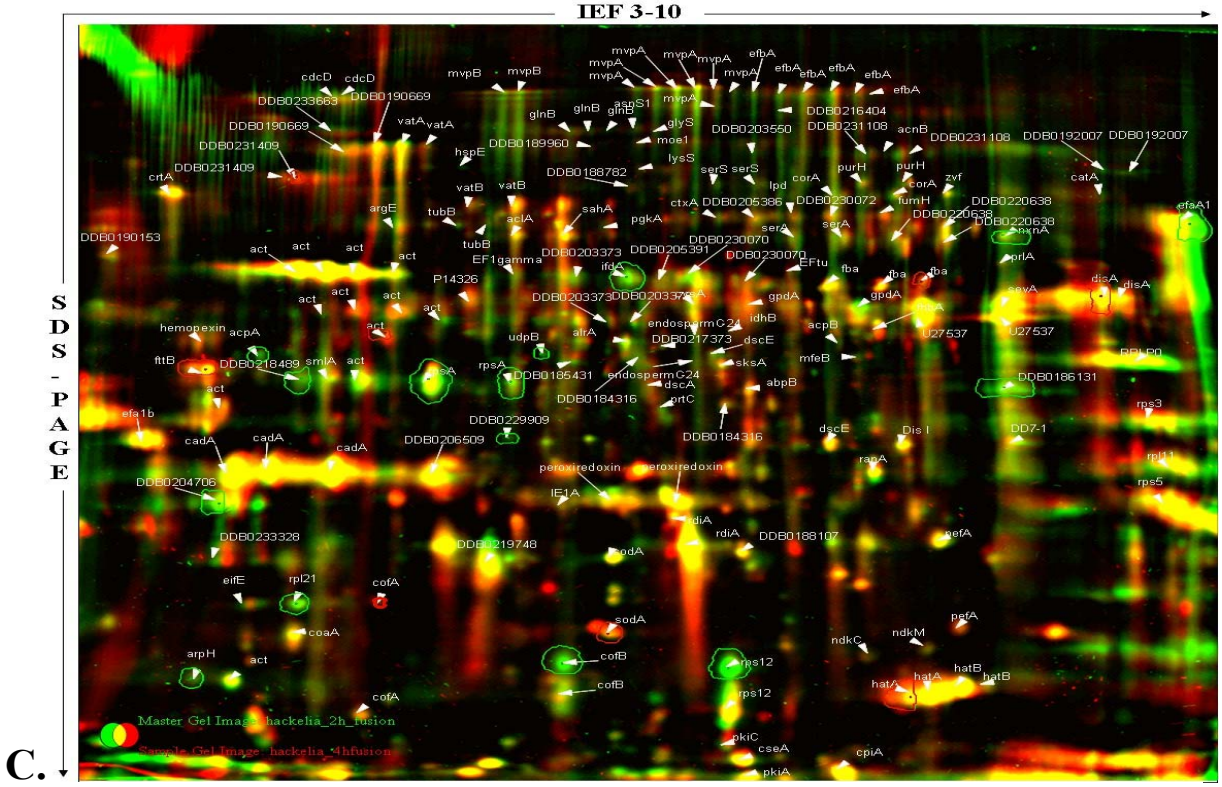
Fig. 8-2 Schematic illustration of *D. discoideum* Pathway "Removal of superoxide radicals"
***D. discoideum* reaction 1.11.1.6: $2H_2O_2 \leftrightarrow 2H_2O + O_2$**
***D. discoideum* reaction 1.15.1.1: $2O^- + 2H^+ \leftrightarrow H_2O_2 + O_2$**


IEF 3-10
SDS-PAGE
Master Gel Image: Zym Corby_2h
Sample Gel Image: Zyn Corby_4h

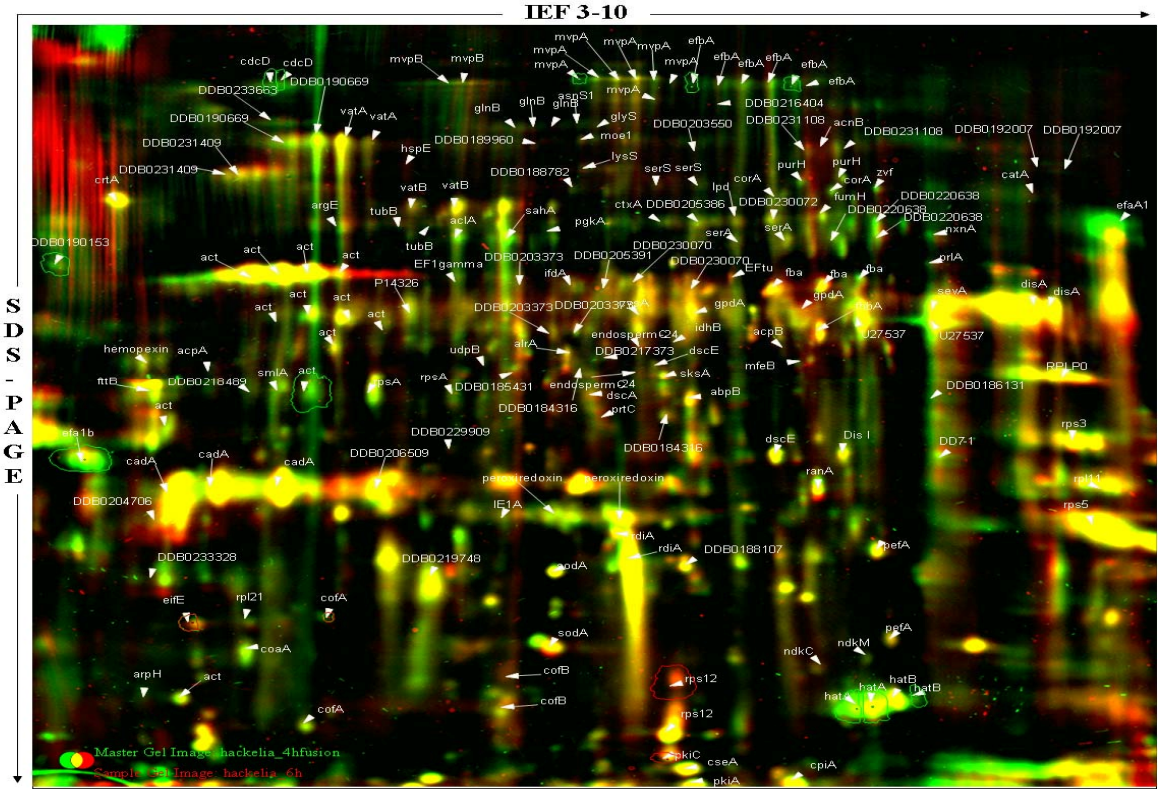
A.


IEF 3-10
SDS-PAGE
Master Gel Image: Zyn Corby_4h_fusion
Sample Gel Image: Zyn Corby_6h_fusion

B.



C.



D.

Fig. 8-3 Dual channel images of the phagosomal protein patterns
The phagosomal proteins were isolated at 2, 4 and 6 hours after infection of *D. discoideum*. Figures A and B represent comparison of 2 hours/4 hours and 4 hours/6 hours of *L. pneumophila* Corby infection, respectively. Figures C and D represent comparison of 2 hours/4 hours and 4 hours/6 hours of *L. hackeliae* infection, respectively.

9 Curriculum vitae

Olga Shevchuk

PERSONAL INFORMATION:

Date of Birth: December 03, 1980
Place of Birth: Krasnoyarsk, Russia
Citizenship: Russian

EDUCATION:

University study	Master of Science
1997-2002	Major subject: Biochemistry
	Department of Biochemistry
	Krasnoyarsk State University, Russia
1999-2000	Practica in Medicine Genetic Center of Krasnoyarsk, laboratory of prenatal diagnostics
	Theme: Level of phenylalanin depending on forms of phenylketonurea and diet therapy
2000-2002	Biology Educational Centre, Institute of Protein Research.
	Russian Academy of Sciences, Pushchino, Moscow Region, Laboratory of genetic engineering
	Title of the Master thesis: Investigation of the role of site-specific endonucleases in exchange of genetic information between T5-like phages.
Doctoral Studies 2003-2008	Doctoral research work at Institute for Molecular Biology of Infectious Diseases, University of Würzburg, and at Institut for Microbiology, Technical University of Braunschweig, Germany
	Title of the Ph.D. thesis: *D. discoideum* as a model for host-pathogen interaction: Phagosomal proteome of *Legionella*-infected cells

PRESENTATIONS AND WORKSHOPS:

O. Shevchuk, S. Killinger, C. Buchrieser, K. Heuner, M. Steinert (2004). Microarray-based identificationof pathogenic and genetically engineered bacteria. International Symposium "Threat of Infection" Würzburg, Germany. Poster presentation.

O. Shevchuk (2005). Isolation and characterization of *Legionella*-containing phagosomes. *Legionella* meeting, Berlin, Germany. Oral presentation.

O. Shevchuk, K. Heuner, C. Batzilla, C. Buchrieser, M. Steinert (2005). Transcription analysis of *Legionella pneumophila* regulators and proteome of the *Dictyostelium* host phagosome. Workshop „Molecular Microbial Pathogenesis", European graduate college, Umeå, Sweden. Poster presentation.

O. Shevchuk, G. Glöckner, K. Heuner, M. Steinert (2005). Development of a custom tailored diagnostic DNA-array for *Legionella*. The 2nd European Conference on Prokaryotic Genomes (PROKAGEN 2005) and the DGHM (Deutsche Gesellschaft für Hygiene und Mikrobiologie eV), Göttingen, Poster presentation.

O. Shevchuk (2005). Host-pathogen interaction: characterisation of *Legionella*-containing phagosome. The 1st International Symposium "Cell Fate Decisions in Health and Disease", Würzburg, Germany. Oral presentation.

O. Shevchuk, M. Steinert (2008). *D. discoideum* mechanical screening for selection of *Legionella* mutants defective in host cell phagosomal maturation. International conference "Functional genomics of microorganisms, Institut Pasteur, Paris. Poster presentation.

PUBLICATIONS:

O. Shevchuk and M. Steinert (2008) Screening of virulence traits in *Legionella pneumophila* and analysis of the host susceptibility to infection by using the *Dictyostelium* host model system. Methods Mol Biol. In press.

M. Steinert, C. Wagner, M. Fajardo, **O. Shevchuk,** C. Ünal, F. Galka, K. Heuner, L. Eichinger and S. Bozzaro (2006) The amoeba *Dictyostelium discoideum* contributes to *Legionella* infection. International conference on *Legionella. Legionella*: state of the art 30 years after its recognition. Edited by N.P. Cianciotto et.al.

O. Shevchuk, C. Batzilla, S. Hägele, S. Engelmann, A. Haas, K. Heuner, G. Glöckner, M. Steinert (2008) First proteomic analysis of a pathogen-containing phagosome reveals *Legionella*-specific expression patterns in *Dictyostelium*. Cellular Microbiology. Submitted.